



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> METHOD OF ASSAY OF INHIBIN  <b>(57) Abstract</b>  A method of immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin. Preferably, the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof. The assay may suitably be a radioimmunoassay, a fluorescence-based immunoassay, or an enzyme-linked immunosorbent assay using labelled 58kD or 31kD inhibin as tracer. Tracers and standards for use in the assay are described and claimed.		

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#### METHOD OF ASSAY OF INHIBIN

5           This invention relates to methods for assay of inhibin, and in particular to methods for immunoassay of inhibin.

#### Background Art

          Two forms of inhibin from bovine follicular fluid  
10 have been recently purified to homogeneity, with molecular masses of 58kD and 31kD (International Patent Application PCT/AU/85/00119 and Robertson et al 1985, 1986). Under  
reducing conditions both forms consist of two subunits with molecular masses of 43kD and 15kD, and 20kD and 15kD  
15 respectively. Their primary amino acid structures have been

elucidated following cloning and analysis of cDNA species derived from bovine granulosa cell mRNA (International Patent Application PCT/AU86/00097; Forage et al 1986). These studies indicate that 31kD inhibin is a processed form of the 58kD molecule. 31-32kD inhibin molecules with similar subunit structures to bFF inhibin have been isolated from porcine follicular fluid (Miyamoto et al 1985, Ling et al 1985) and sequenced (Mason et al 1985).

Currently inhibin activity is measured by a variety of in vivo and in vitro bioassay systems (Baker et al 1981). These systems are time consuming, expensive, have limited sensitivity and precision and are of limited practicability in their application to large sample numbers (Baker et al 1981, Lee et al 1982).

#### 15 Summary of the Invention

The present invention relates to more convenient assays for the estimation of inhibin than have heretofore been possible. The preferred assays of the invention are radioimmunoassays and the following description, whilst being directed to the preferred assays, should not be construed as limiting the invention to radioimmunoassays. Other assays within the scope of the invention include ELISAs, immunoassays based on fluorescence detection, and related assays relying on polyclonal and monoclonal antibodies against inhibin.

#### 25 Preferred Embodiments of the Invention

According to one aspect of the present invention there is provided an immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin.

30 Preferably the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof. Particularly preferred antigens include preparations containing 35 inhibin, purified bovine 58kD inhibin,

purified bovine 31kD inhibin, human inhibin, or human or bovine inhibin or fragments thereof produced using recombinant DNA technology.

Suitable animals include mammals such as mice, rabbits, horses, donkeys, dogs, sheep, and goats, and birds such as chickens.

Alternatively a monoclonal antibody or an IgG directed against any of the aforesaid inhibins may be used.

Most preferably the antibody is capable of neutralizing inhibin bioactivity.

Preferably the immunoassay is further characterized by the step of using labelled 58kD or 31kD inhibin as tracer. More preferably said tracer is labelled with  $^{125}\text{I}$  iodine ( $^{125}\text{I}$ ) with an enzyme, or with a fluorescent marker.

Preferably the assay is a radioimmunoassay or an enzyme-linked immunosorbent assay (ELISA), or a fluorescence-based immunoassay.

15           The invention provides a method for measuring inhibin in samples such as follicular fluid or serum from various species (including humans) wherein concentrations of inhibin in standards are used to derive the concentration of inhibin in the follicular fluid or serum by competitive  
20 binding of  $^{125}\text{I}$  labelled inhibin and inhibin from test samples with bovine 58kD inhibin antiserum, followed by precipitation and counting of bound  $^{125}\text{I}$  labelled inhibin.

          The preferred specific radioimmunoassay system for inhibin of the invention is applicable to bovine and human  
25 follicular fluid and serum, and can employ an antiserum against (purified bovine) 58kD inhibin with iodinated 31kD or 58kD inhibin as tracer.

          According to a second aspect of the invention, there is provided a method for preparation and purification of  
30  $^{125}\text{I}$ -labelled inhibin tracer which comprises the steps of iodination of inhibin using a Chloramine T procedure and purification of  $^{125}\text{I}$ -inhibin by an affinity fractionation step.

          Preferably the affinity fractionation step uses  
35 Matrex Red A.

          Preferably the purification procedure additionally comprises a gel filtration step.

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According to a third aspect of the invention there is provided an assay standard selected from the group consisting of naturally-occurring or recombinant inhibin, or fragments or derivatives thereof. Preferably the standard displays parallelism in the assay with the samples under test.

Particularly preferred standards include bovine 31kD inhibin, and partially purified or purified human inhibin.

The conditions of the assay, in particular incubation times, may be varied in order to attain desired levels of sensitivity.

A form of the radioimmunoassay modified for increased sensitivity comprises:

incubating sample and antiserum for 4 days at 4°C, followed by the addition of  $^{125}\text{I}$ -31kD inhibin tracer, incubating for 3 days at 4°C and then adding second antibody, precipitating, and counting bound  $^{125}\text{I}$  labelled inhibin.

According to one particularly preferred embodiment, suitable for measurement of inhibin in human serum samples, the tracer is  $^{125}\text{I}$ -31kD inhibin, and incubation with tracer is performed at elevated temperature (30°C) in the presence of inhibin-free serum, in order to minimize non-specific effects. Suitable sources of inhibin-free serum include steers or other castrated male animals, oophorectomized women, women with premature ovarian failure, and post-menopausal women.

## 25 Brief Description of the Drawings

Figure 1 shows the fractionation of  $^{125}\text{I}$ -58kD and  $^{125}\text{I}$ -31kD inhibin on analytical SDS-PAGE under reducing conditions.

Figure 2 shows the time course of immunization of a rabbit with 58kD inhibin.

Figure 3 shows the in vitro neutralization of bFF inhibin by an antiserum raised to 58kD inhibin.

Figure 4 shows the radioimmunoassay dose response curves of bFF, hFF, purified 58kD and 31kD inhibin and bovine granulosa cell culture medium (BGCM) using either  $^{125}\text{I}$ -31kD or  $^{125}\text{I}$ -58kD inhibin as tracers.

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Figure 5 shows the profile of inhibin in vitro bioactivity and immunoactivity following fractionation of bFF through the various steps of the inhibin purification procedure of Robertson et al (1986).

5        Figure 6 shows non-reduced SDS-PAGE profiles of  $^{125}\text{I}$ -58kD and  $^{125}\text{I}$ -31kD inhibin following incubation with bFF and serum under conditions used in the RIA of serum inhibin.

Figure 7 shows the effect of temperature on the binding of  $^{125}\text{I}$ -31kD inhibin to the antiserum.

10       Figure 8 shows logit-log dose response lines of bovine and human serum, in the plasma RIA system employing  $^{125}\text{I}$ -31kD inhibin as tracer.

Figure 9 shows the ovulation induction regime and serum levels of FSH, LH, inhibin and oestradiol ( $\text{E}_2$ ) in 15 twenty-six women involved in an In Vitro Fertilisation (IVF) programme and one normal woman (FL#27).

Figure 10 is a comparison of plasma  $\text{E}_2$  and inhibin levels plotted for some of the data in Figure 9.

Figure 11 shows the correlation between the number 20 of ova produced and  $\text{E}_2$  or inhibin levels in serum.

Figure 12 shows the correlation between the numbers of ovarian follicles detected ultrasonically and peak inhibin levels in serum.

Figure 13 shows inhibin, FSH, progesterone and 25 oestradiol concentrations in the sera of non-pregnant subjects ( $n = 16$ ) on the days following oocyte retrieval.

Figure 14 shows inhibin, FSH, progesterone and oestradiol concentrations in the sera of pregnant subjects ( $n = 3$ ) on the days following oocyte retrieval.

30       Figure 15 shows the relationship between serum inhibin and FSH during the luteal phase of non-pregnant subjects. For this analysis, non-detectable inhibin values ( $n = 29$ ) were assigned the limit of assay sensitivity.

Figure 16 shows inhibin, FSH, LH, oestradiol and progesterone concentrations in the sera of normal women during the menstrual cycle, assayed using anti-31kD inhibin.

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Detailed Description of the InventionAbbreviations

	bFF	:	bovine follicular fluid
	hFF	:	human follicular fluid
5	oFF	:	ovine follicular fluid
	oRTF	:	ovine rete testis fluid
	HPLC	:	high performance liquid chromatography
	SDS-PAGE:		sodium dodecyl sulphate
			polyacrylamide gel electrophoresis
10	RIA	:	radioimmunoassay
	SS	:	steer serum
	BS	:	bull serum
	CS	:	cow serum
	PMS	:	human post-menopausal serum
15	HFP	:	human female plasma

Preparations(a) Purification of bFF inhibin

The purification of bFF 31kD and 58kD inhibin was based on the procedures described previously (Robertson et al 1985, 1986) (Figure 5):

(a) bFF was fractionated on a Sephacryl S200 (9 x 90 cm) gel filtration column in 0.05M ammonium acetate pH 7.0.

(b) The void volume fraction from (a) was precipitated at pH 4.75 and fractionated on Sephadex G100 (9 x 90 cm) in 4M acetic acid.

(c) and (d)

Peak I (58kD inhibin) and Peak II (31kD inhibin) fractions from (b) were fractionated on an RPSC Ultrapore column (0.46 x 7.6 cm, Beckman) using a 0-50% acetonitrile gradient in 0.1% trifluoroacetic acid. In Figure 5 the continuous line indicates optical density at 280 nm (a) and (b) and 254 nm (c) and (d).

Hatched area denotes inhibin bioactivity.  
o---o RIA with <sup>125</sup>I-58kD inhibin as tracer.



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o\_\_o RIA with  $^{125}\text{I}$ -31kD inhibin as tracer.

$V_o$  = void volume.

BSA = bovine serum albumin (mol. wt 67,000).

OVA = ovalbumin (mol. wt 43,000).

- 5       The purified inhibin was stored in SDS electroelution buffer (approx. 3% SDS in 10 mM  $\text{NH}_4\text{HCO}_3$ ) prior to iodination. For bioassay, samples were methanol precipitated at  $-20^\circ\text{C}$  in order to remove SDS and solubilized by heating at  $37^\circ\text{C}$  for 1 hour and sonication.
- 10       Similar profiles of both bio- and immunoactive inhibin were observed at each stage of the inhibin purification procedure. The biological to immunological activity ratios for a number of purified 31kD and 58kD inhibin preparations using both tracers in the radioimmunoassay ranged
- 15 from 0.30 - 0.43.

#### (b) Sample Preparation

- Human follicular fluid was obtained at oocyte collection in the in vitro fertilisation programme at the Queen Victoria Medical Centre/Epworth Hospital, Melbourne. It
- 20 was charcoal treated (100 mg/ml dextran-coated charcoal for 1 hour at  $4^\circ\text{C}$ ), lyophilised, stored at  $-20^\circ\text{C}$  and resolubilized prior to assay by sonication in assay buffer or culture medium. Ovine follicular fluid (oFF) was obtained by aspiration of ovaries collected at a local abattoir. Ovine
- 25 rete testis fluid (oRTF) is a lyophilised inhibin preparation (Baker et al 1985). Rat ovarian extract was a charcoal-treated rat ovarian cytosol preparation. Details of the biopotencies of these inhibin preparations are outlined in Table 1.

- 30       Blood was collected in lithium heparin tubes from 40 women undergoing ovulation induction therapy (clomiphene citrate and human menopausal gonadotrophin treatment) with plasma estradiol levels at time of plasma collection ranging from 40-2,900 pg/ml. Equal aliquots of serum from each
- 35 subject were combined to produce a plasma pool designated as

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Human Female Plasma pool (HFP). Plasma from four post-menopausal women aged 52 and over were combined to give a pool designated Post-Menopausal Serum pool (PMS).

Bovine blood, ovaries and testes were collected on ice from a local abattoir and processed within one hour. All samples were stored at  $-20^{\circ}\text{C}$  after snap freezing in solid  $\text{CO}_2$ /ethanol. Blood pools from adult intact (BS,  $n = 9$ ) and castrate (SS,  $n = 1$ ) male, and female (CS,  $n = 10$ ) cattle were allowed to clot overnight at  $4^{\circ}\text{C}$  prior to centrifugation and storage. Bovine ovarian follicles were hemisected and granulosa cells were collected by aspiration and cultured for 40 hours at a concentration of  $10^5$  viable cells per well (Costar 48 well plate) in 400  $\mu\text{l}$  DMEM/F12 complete medium. Media were stored frozen at  $-20^{\circ}\text{C}$  prior to assay. Testes from four bulls were decapsulated and homogenised in equal w/v Dulbecco's phosphate buffer using an Ultra-Turrax tissue disperser (Janke and Kunkal KS, Staufen FRG) and centrifuged at 100,000g x 1 hour at  $4^{\circ}\text{C}$  and stored at  $-20^{\circ}\text{C}$ . Prior to assay the supernatants were charcoal treated with an equal volume of 1% Norit A in Dulbecco's phosphate buffer and incubated at  $4^{\circ}\text{C}$  for 30 minutes prior to centrifugation and bioassay (Au et al 1983).

#### Immunization Procedure

Purified 58kD inhibin (14  $\mu\text{g}$  in 500  $\mu\text{l}$  Dulbecco's phosphate buffer) was emulsified in an equal volume of adjuvant (Marcol 52 [Esso, Australia]: Montanide 888 [S.E.P.P.I.C., Paris] in the ratio 9:1) and injected into an intact male New Zealand white rabbit 4 intramuscular and one subcutaneous sites. Two booster injections of 14  $\mu\text{g}$  under the same conditions were given at six weeks and one year. Serum was collected throughout for assessment of its in vitro neutralization activity, its ability to bind iodinated inhibin and for plasma FSH estimations.

A similar procedure was used to raise antibody directed against purified 31kD inhibin.

Analytical SDS Polyacrylamide Gel Electrophoresis

Sera and bFF were incubated at various temperatures in an equal volume of 100 mM phosphate buffer pH 7.4 containing 0.15M NaCl, 0.1% Triton X-100 and either 0.5% BSA for studies with  $^{125}\text{I}$ -31kD inhibin or 0.5% Polypep for  $^{125}\text{I}$ -58kD inhibin. Equal volumes (5  $\mu\text{l}$ ) of sample and 10% SDS and Dulbecco's Phosphate buffer pH 7.4 (30  $\mu\text{l}$ ) were placed in a boiling water bath for 2.0 minutes then in ice. Ten microlitres of bromophenol blue (0.006%) in glycerol (62.5% in  $\text{H}_2\text{O}$ ) was then added and the mixture centrifuged prior to electrophoresis on 12.5% slab gels (3 hours, 20-30 mA). Protein molecular weight markers were either co-electrophoresed with the iodinated sample in the absence of bFF and serum or on a separate track in their presence. The gels were fixed and stained overnight in ethanol:  $\text{H}_2\text{O}$  formaldehyde (180: 420: 100) containing 0.1% Coomassie Brilliant Blue. Each track was divided into 50 2mm slices and counted in a gamma counter.

Reversed-phase HPLC

$^{125}\text{I}$ -Inhibin was applied to an Ultrapore RPSC column (0.46 x 7.5 cm, Beckman, Berkeley, Ca., USA) and fractionated using a 30 min linear gradient of 0.50% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min and 0.5 ml fractions using Waters HPLC apparatus (model 6000A pumps and a model 660 Programmer, Milford, Mass., USA).

In Vitro Bioassay

Inhibin activity was determined using an in vitro bioassay based upon the dose-dependent suppression of FSH cell content in rat pituitary cell cultures utilizing a parallel line bioassay design (Scott et al 1980). The charcoal-treated bovine follicular fluid preparation employed a lymph reference preparation with an arbitrary unitage of 1 unit/mg (Scott et al 1980).

Hormone Assays

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(i) Rabbit FSH Radioimmunoassay

Rabbit FSH was determined using an RIA kit kindly provided by Dr. A.F. Parlow (Torrance, Ca, USA) employing 15% polyethylene glycol to separate bound and free hormone. The sensitivity of the assay was 0.9 ng/ml using rabbit FSH AFP.538.C as standard. The within assay coefficient of variation was 8.1% and all samples were assayed in the one assay.

(ii) Rat FSH Radioimmunoassay

Rat FSH generated by the pituitary cells in culture was measured by a specific radioimmunoassay using reagents supplied by the NIAMDD.  $^{125}\text{I}$ -rat FSH ( $\text{I}_5$ ) was used as tracer and FSH RP-2 used as standard. The within-assay coefficient of variation was 7%.

(iii) RIA of human hormones

Serum FSH was measured by RIA (Amerlex-M, Amersham, USA) using 2nd IRP FSH as standard with an interassay CV of 7.0% from 31 assays. LH was measured by RIA (LH RIA, Diagnostic Products Corp., L.A., USA) using the 2nd IRP LH as standard with an interassay CV of 10.1% from 31 assays. Both oestradiol and progesterone were measured using RIA (Coat-a-Count, Diagnostic Products Corp., L.A.) with interassay CVs of 8.7% and 8.1% respectively from 150 assays. Serum beta subunit of hCG was measured by RIA (B-hCG RIA-Quant, Mallinckrodt Inc. St. Louis, USA) using the hCG 2nd IS as standard with an interassay CV of 10.4% from 30 assays.

Calculations

The RIA dose-response curves were linearised using a logit-log dose transformation. Parallelism was assessed from a comparison of slope values of dose-response curves using the multiple range test for groups of unequal size (Kramer, 1956) or by paired t-test. Potency estimates were determined using standard parallel line bioassay statistics. In situations where non-parallelism was observed between dose response lines

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of unknown and standard preparations, potency estimates were determined from the ratio of their  $ED_{50}$  values. The sensitivity ( $ED_{10}$ ) was defined as the mass of hormone required to give 10% displacement in the assay whilst  $ED_{50}$  corresponded to the mass required for 50% displacement.

The index of precision (Gaddum (1933); Finney (1964)) was used to describe assay precision. The between assay variation was calculated from the coefficient of variation of the repeated measurement of a partially purified inhibin preparation. The dissociation constant ( $K_{dis}$ ) was determined by Scatchard analysis using  $^{125}I$ -hormone and increasing amounts of unlabelled hormone. The mass of  $^{125}I$ -hormone used in the analysis was determined from its specific activity ( $\mu Ci/\mu g$ ).

The invention will now be illustrated by reference to the following non-limiting examples.

#### Example 1      Antiserum Characterization

Antisera against 58kD and 31kD inhibin were characterized by showing that following immunization, parallel changes in plasma FSH and inhibin antibody titre were observed, indicating inhibin neutralization in vivo. The antisera neutralized bFF, hFF and purified 31kD and 58kD inhibin activity in an in vitro bioassay. The results described below refer to anti-58kD inhibin, but similar results were obtained using anti-31kD inhibin.

##### (a)      Response In Vitro to Immunization

Following the first booster (Fig. 2a) the antibody titre was assessed by the ability of the antibody to neutralize inhibin bioactivity in vitro and its capacity to bind  $^{125}I$ -58kD inhibin. A sharp parallel elevation in these activities was observed between 1-8 weeks post-booster injection. During this period significant ( $P < 0.05$ ) elevation in serum FSH ( $6.63 \pm 0.95$  ng/ml,  $n = 5$  vs  $4.97 \pm 0.87$  ng/ml,  $n = 6$  (mean  $\pm 1$  SD)) was noted. Following the second booster (Fig. 2b), an immediate and sustained elevation of serum  $^{125}I$ -31kD inhibin-binding capacity and serum FSH was observed.

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Basal levels of serum FSH were assessed from the mean + 2 S.D. of 14 observations over the preceeding five months (hatched area Figure 2b).

These results indicate that purified inhibin from bovine follicular fluid can be used to immunize rabbits, producing an antiserum which has the capability of neutralizing inhibin bioactivity. The elevated levels of plasma FSH in the rabbit observed during the period of peak antiserum titre (as assessed by iodinated inhibin binding and in vitro neutralizing capacity) indicate that the antibodies produced are capable of neutralizing endogenous inhibin. The combination of the neutralization of inhibin both in vivo and in vitro and the close relationship of the neutralizing activity and iodinated inhibin binding capacity of the antiserum provides convincing evidence of its specificity.

#### (b) Inhibin Neutralisation In Vitro

Bovine follicular fluid inhibin (2 units) was quantitatively neutralised by 1  $\mu$ l antiserum per culture well while 75% inhibition of bioactivity was achieved with 0.35  $\mu$ l per well (Fig. 3, Table 1). In Figure 3, the vertical dotted line indicates the volume of antiserum required to achieve 75% neutralization of inhibin activity, an arbitrary parameter of antiserum neutralizing titre. Purified 58 and 31kD inhibin gave corresponding 75% inhibition values of 0.33 and 0.38  $\mu$ l respectively. In comparison, neutralisation of hFF inhibin bioactivity required 1.32  $\mu$ l antiserum (n = 2, Table 1) corresponding to 27% cross-reactivity in comparison to bFF inhibin. Inhibin from ovine sources (follicular fluid, rete testis fluid) showed 8 and 6% cross-reactivity respectively.

This antiserum, at a maximum non-toxic level of 4  $\mu$ l per well, did not neutralise 2 units of inhibin activity in rat ovarian cytosol extracts.

TABLE 1

Cross-reactivity of inhibin from various sources as assessed by inhibin neutralization in vitro and radioimmunoassay

IN VITRO NEUTRALIZATION			RADIOIMMUNOASSAY			
Preparation	125I-58kD Inhibin		125I-31kD Inhibin			
	Inhibin Bioactivity U/ml	Antiserum Titre ( $\mu$ l)	% Cross-Reactivity	ED <sub>50</sub> (U)	% Cross-Reaction	% Cross-Reaction
<u>Follicular Fluid</u>						
Bovine	5200	0.35 $\pm$ 0.04 (6)	100	1.30 $\pm$ 0.25 (9)	100	100
Human	168 $\pm$ 32 (6)	1.32 (2)	27	5.3 $\pm$ 1.6 (4)	28.4 $\pm$ 8.7 (4)	37
Ovine	27,000 (2)	4.4 (2)	8.0	> 320	< 0.3	< 0.1
<u>Ovarian Extract</u>						
Rat	384 (2)	> 6 (2)	< 6.0	**	**	
<u>Rete Testis Fluid</u>						
Ovine	1040*	> 6.0 (1)	< 6.0	> 52	< 2	< 2

\* U/mg protein

\*\* No displacement with 16 U inhibin  
Mean  $\pm$  SD (n)

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Example 2 Iodination of inhibin

Iodination of either 58kD or 31kD inhibin has been achieved using a conventional Chloramine T iodination procedure and was associated with considerable iodination damage. Purification of the tracer was therefore necessary and it was not achieved following gel filtration chromatography on Sephadex G25. Specific binding of either iodinated hormone to Matrex Red A achieved satisfactory purification although recoveries were low. Either iodinated inhibin form thus purified had the physico-chemical properties of its non-iodinated form.

Alternative iodination procedures using Iodogen, Iodobeads, lactoperoxidase, or Bolton-Hunter reagent were found to cause less damage to the inhibin molecule, but resulted in poorer incorporation of radioactivity. Consequently iodination using Chloramine T was preferred.

Purified 58kD or 31kD inhibin (1-2 µg in 25 µl electroelution buffer was added to 25 µl 0.5 M phosphate buffer, pH 7.2. Na<sup>125</sup>I (0.5 mCi, 5 µl; Amersham, Bucks, UK) was added. Chloramine T (40 µl) was added at a ratio of 8:1 Chloramine T to hormone. The reaction proceeded for 60 seconds at room temperature with stirring and was terminated with 20 µl sodium metabisulphite (3 mg/ml). The reaction mixture was made up to 50 µl in 20mM phosphate buffer 0.1% BSA or 0.5% Polypep (Sigma, St. Louis, Mo., USA) pH 6.0 and gel filtered on a Sephadex G25 column (PD10, Pharmacia, Uppsala, Sweden) to remove <sup>125</sup>I. The void volume fractions were pooled, made up to 20 ml and applied to a column of 200 µl Matrex Red A (Amicon, Danvers, Mass., USA) and then washed with phosphate buffer containing 400 mM KCl, the eluted counts being discarded. <sup>125</sup>I-inhibin was eluted with 1M KCl/4M urea in phosphate buffer. The iodinated inhibin was further gel filtered on a Sephadex G25 column (PD10) with the appropriate RIA buffer (see below) to remove the KCl/urea.

Following iodination of 58kD and 31kD inhibin, 60 µCi and 25 µCi respectively were recovered in the void volume fractions following gel chromatography on Sephadex G25.



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Approximately 30% was eluted with the 1M KCl/4M urea buffer.  $^{125}\text{I}$ -inhibin, as assessed by its molecular weight on SDS-PAGE, was found in this fraction.

The specific activity of the iodinated preparations was assessed in the radioimmunoassay using a self-displacement procedure (Marana *et al* 1979) with the hormone used for iodination as standard. Specific activities of 50-60  $\mu\text{Ci}/\mu\text{g}$  for 58kD inhibin and 24  $\mu\text{Ci}/\mu\text{g}$  for 31kD inhibin were obtained, with recoveries ranging from 5-25%.

10 Example 3 Characterisation of iodinated inhibin

The physico-chemical characteristics of  $^{125}\text{I}$ -inhibin were assessed using RP-HPLC and SDS-PAGE. A close correspondence was observed between the radioactive and bioactive profiles on RP-HPLC for both the 58kD and 31kD preparations (data not shown). The molecular mass of  $^{125}\text{I}$ -58kD inhibin following fractionation on SDS-PAGE was similar to purified non-iodinated inhibin under both non-reducing (58kD) and reducing (43kD and 15kD) conditions except that a 58kD material of unknown identity was observed in relatively low proportions (18%) under reducing conditions (Fig. 1). The molecular weight markers employed were BSA (bovine serum albumin) 67,000; OV (ovalbumin) 43,000; CA (carbonic anhydrase) 29,000; GL (goose egg lysozyme) 20,300; and CL (chick egg lysozyme) 14,300. The arrow, in figure 1, refers to the point of sample application. Radioactivity found in fractions beyond fraction 47 represents free iodine in the solvent front. The purity of the inhibin used for iodination as assessed by silver staining on SDS-PAGE suggests that the  $^{125}\text{I}$ -58kD material is not an iodinated contaminant. In support,  $^{125}\text{I}$ -58kD inhibin was fractionated by microelectrofocusing procedure on the pH range 3.5-10 and 4-8 (Foulds and Robertson 1983), and 3 peaks of radioactivity with pI values of 7.4, 6.2 and 5.2 were observed. Upon reduction on SDS-PAGE each of these peaks showed persistence of  $^{125}\text{I}$ -58kD material. The results suggest that the presence of

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the  $^{125}\text{I}$ -58kD material is attributable to difficulties in reduction of the iodinated hormone rather than to the iodination of a contaminating protein.

Fractionation of the  $^{125}\text{I}$ -31kD inhibin on SDS-PAGE revealed molecular weights of 30,200 under non-reducing conditions and 20,000 and 15,000 subunits following reduction; these values are similar to those for the non-iodinated hormone. A second antibody RIA system using either tracer yielded a parallel displacement between purified 31kD and 58kD inhibin.

#### Example 4 Radioimmunoassay Procedure

The assay buffer used was 10 mM phosphate, 0.15 M NaCl, 0.5% BSA, pH 7.2. A delayed tracer addition, second antibody assay system was employed. The sample and antiserum were incubated in a volume of 300  $\mu\text{l}$  for 16 hours at room temperature following which  $^{125}\text{I}$ -inhibin (10,000 cpm, 100  $\mu\text{l}$ ) was added and the incubation continued either overnight at room temperature or for 48 hours at 4°C. Second antibody (goat antiserum to rabbit IgG, 100  $\mu\text{l}$ ) was added and incubated for 1 hour at 4°C following which 1 ml 6% polyethylene glycol was added. The tubes were vortexed and incubated for a further 30 min, spun at 2000 g for 30 min at 4°C, decanted and counted. The inclusion of Triton X-100 (final concentration 0.025%) in the assay buffer reduced non-specific binding from 4 to 0.5%.

Radioimmunoassay procedures were established using both 31 and 58kD inhibin tracers. Following a logit-log dose transformation of the response curves, linear displacement of each tracer was observed for a range of inhibin preparations, with the exception of 31kD inhibin when using  $^{125}\text{I}$ -58kD inhibin as tracer, in which a deviation from linearity below logit -0.5 (38% B/Bo) was seen (Fig. 4). In figure 4, each value represents the mean  $\pm$  SD of triplicates. The characteristics of each assay are outlined in Table 2. Scatchard analysis revealed similar affinities for the antiserum of either inhibin form. Non-parallel dose response

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lines were observed between bFF and either 31kD inhibin with  $^{125}\text{I}$ -31kD inhibin as tracer of 58kD inhibin with  $^{125}\text{I}$ -58kD inhibin as inhibin tracer. The sensitivity ( $\text{ED}_{10}$ ) and  $\text{ED}_{50}$  values were comparable in each assay with either hormone.

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TABLE 2

Characteristics of the two radioimmunoassay systems with

<sup>125</sup>I-31kD inhibin and <sup>125</sup>I-58kD inhibin as tracers

	<sup>125</sup> I-31kD inhibin	<sup>125</sup> I-58kD inhibin
5		
Antibody Dilution	1:8000	1:4000
Tracer binding (Bo)	30%	18%
Affinity (Kdis) x 10 <sup>-10</sup> M	0.66	0.72
	20°C	
10 ED <sub>10</sub> (ng, fm)		
31kD inhibin	0.10, 3.0	0.13, 4.4
58kD inhibin	0.07, 1.2	0.13, 2.2
ED <sub>50</sub> (ng)		
31kD inhibin	0.30, 10.1	0.51, 17.1
15 58kD inhibin	0.26, 4.3	0.43, 7.0
Slope*		
bFF	1.37 ± 0.09 <sup>a</sup> (8)	1.47 ± 0.09 <sup>c</sup> (8)
31kD inhibin	1.53 ± 0.09 <sup>b</sup> (5)	1.68 ± 0.08 (3)
58kD inhibin	1.50 ± 0.07 (3)	1.73 ± 0.14 <sup>d</sup> (5)
20 Precision**	0.036 (5)	0.038 (5)
Between Assay Variation**	14 (5)	8.5% (5)
Bio/Imm Ratio		
31kD inhibin	0.34 ± 0.09 (16)	0.43 ± 0.13 (4)
58kD inhibin	0.30 ± 0.12 (7)	0.37 ± 0.12 (5)
25 BGCM	0.25 (1)	-

a vs b P &lt; 0.05)

) as assessed by paired t-test

a vs c P &lt; 0.01)

30 Mean ± SD

\* Number in brackets: number of preparations

\*\* Number in brackets: number of assays

BGCM = bovine granulosa cell culture medium

For details see text.

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Example 5    Specificity of the assay

The specificity was assessed on the following grounds. First, a similar hierarchy of cross-reaction of inhibin from various species in the RIA using either tracer and in vitro neutralisation studies was observed (Table 1). The cross-reaction in the radioimmunoassay of inhibin from different species when expressed in terms of their bioactivity was bFF 100%, hFF 30% ovine FF 1% and rat ovarian extract non-detectable. With respect to this antiserum it is apparent that both male and female bovine and human inhibin share common antigenic determinants not found in inhibin from the other two species. This implies close structural similarity between inhibin from both sexes and species. Secondly, no cross-reaction (0.5%) occurred for a range of purified glycoproteins and polypeptides. Rat LH and FSH, ovine LH and FSH, hCG, bovine TSH, LHRH, ovalbumin and bovine serum albumin showed less than 0.5% cross reactivity using either tracer. Alternatively, medium from the bovine granulosa cell culture (Fig. 4), and bovine testis extract (data not shown), both containing inhibin bioactivity, gave parallel displacement curves to bFF inhibin in the RIA. The parallel dilution of inhibin bio- and immunoactivity of medium from bovine granulosa cell culture with the inhibin standard provides evidence for these cells being the site of inhibin production, as has been previously suggested (Erickson and Hsueh 1978; Henderson and Franchimont 1981). Thirdly, similarities were observed in the profiles of both biological and immunological activities following fractionation of bFF on gel filtration chromatography and RP-HPLC. However, in the 40-60kD molecular mass region of the Sephacryl S200 column (Figure 5a), an 8-40 fold excess of immunoactivity over bioactivity was present, accounting for 12-18% of the recovered immunoactivity.

A large variation in the ratio of biological/immunological activities with charcoal-treated bFF as standard was observed following fractionation of bFF inhibin on gel filtration and RP-HPLC (Fig. 5) and between purified inhibin preparations (Table 2). The ratios ranged

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from 0.02-2.09 in fractions obtained during the purification procedure and from 0.30-0.43 with the purified inhibin preparations.

It is concluded that the RIA procedures are not detecting molecular entities devoid of biological activity and vice versa except in the lower molecular weight region (40-60kD) of the Sephacryl S200 chromatogram. Whether this lower molecular weight material represents a protein distinct from inhibin which cross-reacts in the RIA or inhibin devoid of biological activity has not been established.

The cross-reactivities of inhibin-related proteins in the RIA relative to 31 kDa bFF inhibin were as follows: porcine transforming growth factor-beta (R & D Systems, Minn. USA) <0.9%, bovine Mullerian inhibitory substance (kindly provided by Dr. J. Hutson, Royal Children's Hospital, Melbourne) <0.3% purified bovine inhibin B subunit dimer <2% and the subunits of 31 kDa bFF inhibin obtained following reduction and alkylation <0.1%. A range of glycoproteins and growth factors have been previously tested (McLachlan et al, 1986) and showed cross reactivities against anti-58kD inhibin of less than 1.0%. The specificity of anti-31kD inhibin was similar, with cross-reactivities of less than 1.0%.

#### Example 6 Application of the Radioimmunoassay to Serum

The RIA in its application to serum required substantial modification. Firstly 100mM phosphate buffer pH 7.4 containing 0.15M NaCl, 0.5% BSA was used, and, because of its stability in serum,  $^{125}\text{I}$ -31kD inhibin was preferred to  $^{125}\text{I}$ -58kD inhibin as RIA tracer. Secondly, a temperature-dependent interference of steer serum with  $^{125}\text{I}$ -31kD inhibin binding to the antiserum was observed, with an increase in binding (B/Bo) from 57% at 4°C to 94% at 37°C (Fig. 7). This figure demonstrates the temperature dependence following a 16 hour incubation in the presence of various serum and inhibin preparations (bFF, 31kD inhibin, steer serum (SS), cow serum (CS), bull serum (BS), human post-menopausal serum (PMS), human female serum pool (NFP)). Tracer binding (B/T) was maximal at 30°C in the presence of steer serum, being  $87.1 \pm 3.4\%$  (n = 5). Displacement of  $^{125}\text{I}$ -31kD inhibin

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by bFF or 31kD inhibin was largely unaffected by temperature. Human PMS showed no temperature related interference upon binding although the binding was elevated (B/Bo 110-120%).

Based on these data, conditions for the assay of either bovine or human serum inhibin were established. These involved using  $^{125}\text{I}$ -31kD inhibin as tracer in an overnight 30°C incubation and, in order to compensate for the low level of interference by SS or PMS (presumed to contain no inhibin) standards and samples were diluted in SS or PMS accordingly.

No detectable activity was determined in steer or in human post-menopausal serum, whilst bull and human female serum showed parallel dose-response curves to their respective follicular fluid standards, with circulating levels of 0.9 and 1.1 ng respectively.

Inhibin preparations or serum samples were diluted in SS or PMS to a sample volume of 200 µl. Antiserum (100 µl, final dilution 1:8000) and samples (200 µl) were incubated for 4 hours at 30°C, followed by a further incubation of 16 hours at 30°C in the presence of tracer. Second antibody was added and the tubes were incubated for 24 hours at 4°C, following which 2 ml 0.15M NaCl was added and the tubes were centrifuged.

Thirdly, with respect to a choice of standard in the RIA of bovine serum inhibin, purified 31kD inhibin is favoured in view of its stability in serum. In the absence of a purified human inhibin preparation, 31kD bovine inhibin may be used as the standard in the RIA of human serum inhibin. However, the partially purified hFF inhibin preparation described above is preferred, and purified hFF inhibin, when available, would be the most preferred standard. The detectable levels of inhibin immunoactivity in serum from women under going ovarian stimulation with exogenous gonadotrophin is analogous to the findings of Lee et al (1982), where circulating levels of inhibin activity were detected in PMSG treated immature female rats, particularly directed against 58kD and 31kD inhibin.

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Individual antisera may behave differently in the assay, and assay parameters may have to be determined for each case. Considerable variations in sensitivity between antisera have been observed, particularly between antisera directed against 31kD and 58kD inhibin. Anti-31kD inhibin appeared to give greater sensitivity than anti-58kD inhibin in the samples tested so far.



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Example 7    Improved Sensitivity RIA for human serum

In order to improve assay sensitivity, the assay procedure above was modified as follows: the total volume of the assay was reduced from 400 to 300  $\mu$ l (comprising 200  $\mu$ l sample, 50  $\mu$ l tracer and 50  $\mu$ l antiserum). The assay buffer was 150 mM phosphate, 0.2% BSA pH 7.4, and the incubation of sample and antiserum was 4 days at 4°C followed by the addition of tracer and a further 3 days at 4°C prior to the addition of second antibody. Using this method, a 2.5 fold increase in sensitivity was achieved. This modified assay procedure has been applied to the measurement of human plasma inhibin. The modified assay allows the quantification of plasma inhibin in normal male plasma and in plasma throughout the normal menstrual cycle.

15 Example 8    Stability of  $^{125}$ I-31kD and  $^{125}$ I-58kD Inhibin in Serum

SDS-PAGE profiles of  $^{125}$ I-58kD inhibin following overnight incubation with serum (SS and PMS) showed an increased formation of an  $^{125}$ I-30kD component (12% of recovered activity at 4°C; 17% at 30°C) in comparison with either buffer or bFF (6% 4°C and 30°C; (Fig. 6). The tracers were incubated overnight at either 4 or 30°C with either bFF, steer serum (SS) or human post-menopausal serum (PMS). Incubation of either tracer in RIA buffer alone gave similar profiles to the bFF incubation shown. Molecular weight markers are described in Figure 1. No radioactivity was observed between the position of the marker carbonic anhydrase and the solvent front with either tracer. Results are presented as the mean  $\pm$  SD of three replicate experiments. In contrast, SDS-PAGE profiles of  $^{125}$ I-31kD inhibin under the same incubation conditions showed no significant changes. Recoveries of radioactivity with either tracer were not affected by either temperature or by the presence of bFF or serum.

Example 9 Radioimmunoassay of inhibin in bovine serum

The application of the inhibin RIA to serum from cattle resulted in parallel logit-log dose response lines of BS with either bFF or 31kD inhibin as standards (Fig. 8). The response shown in figure 8 is for bovine and human serum, diluted in steer serum or post-menopausal serum respectively, in the plasma RIA system employing  $^{125}\text{I}$ 31kD inhibin as tracer. Potential RIA standards (bFF, 31kD inhibin, hFF) were assayed in the presence of either phosphate buffer (200  $\mu\text{l}$ , o) or 10 steer serum (200 $\mu\text{l}$ , ●) or post-menopausal serum (200 $\mu\text{l}$ , ▲) and their logit plots were calculated using their respective non-specific binding and  $B_0$  values. Cow serum shows a minimal detectable immunological response. When the immunoactivity was expressed in terms of 31kD inhibin standard the level of 15 inhibin in BS was  $0.91 \pm 0.27$  ( $n = 3$ ) mg/ml and CS 0.1 ng/ml. A parallel response line of HFP was observed in the RIA with 31kD inhibin and hFF as standards corresponding to levels of  $1.05 \pm 0.07$  ( $n = 3$ ) ng/ml. The levels in normal plasma ( $n = 8$ ) were equal to or less than the sensitivity of the assay 20 (0.1 ng/ml).

Example 10 Radioimmunoassay of inhibin in infertile human subjects

The radioimmunoassay previously described was applied to plasma and serum from post-menopausal subjects 25 (presumed to be inhibin free,  $n = 8$ ) and young women with ovarian failure (premature ovarian failure  $n = 2$ , Turner's syndrome  $n = 1$ , ovariectomized  $n = 1$ ). There was no difference in  $^{125}\text{I}$ -31kD inhibin binding in the assay between these two groups and therefore post-menopausal serum was used 30 as a diluent in the assay.

Example 11 Radioimmunoassay of inhibin in human serum

The method was applied to:

(a) Plasma from normal women during spontaneous menstrual cycles from the early follicular phase until the 35 time of ovulation ( $n = 2$ , example 'FL' #27, Fig. 9). Inhibin

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immunoactivity was below the limit of assay detection prior to day 13 and its increase correlated with the increase in circulating levels of oestradiol ( $E_2$ ), LH and FSH on day 13 and 14.

5 (b) Inhibin immunoactivity in serum from normal men ( $n = 7$ ) was at the lower limit of assay detection.

(c) Plasma was obtained from 26 unselected women undergoing their fourth cycle of ovulation induction in the in vitro fertilization programme at the Epworth Hospital,  
10 Richmond, Victoria. Briefly this involved the administration of clomiphene citrate 100-150 mg daily on day 5-9 of the menstrual cycle, followed by human menopausal gonadotrophin (hMG) 75-225U daily for the next 5-7 days. Adequate follicular development was assessed by the progressive  
15 increase in plasma oestradiol and by ovarian ultrasound. Ovulation occurred spontaneously if an endogenous LH spike was observed, or in the absence of the latter, ovulation was induced by administration of 5000 IU of human chorionic gonadotrophin (hCG). For an example of a spontaneous  
20 ovulation see 'BU' # 11, Fig. 9k and for an hCG-induced ovulation sample see 'JO' 1, Fig. 9a.

Inhibin immunoactivity in the plasma samples, as defined in terms of the biological activity of a purified 31kD inhibin standard, showed a highly significant correlation with  
25 plasma oestradiol levels (Fig. 10). The correlation coefficient values have been calculated from the total data in figure 9. An example of the correspondence of plasma oestradiol and inhibin during an ovulation induction cycle is seen in example 'BE' # 9, Fig. 9b. There was also significant  
30 correlation between peak plasma oestradiol concentrations and the number of oocytes recovered, and peak plasma inhibin concentrations and the number of oocytes recovered (Fig. 11), as well as a strong correlation between the peak plasma inhibin concentration and the number of ovarian follicles  
35 detected ultrasonically prior to oocyte aspiration (Fig. 12). It is therefore apparent that both plasma inhibin and plasma

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oestradiol are parameters of follicular development and health, and in the majority of cases these show a close correspondence.

In certain examples ('BY' # 6, 'BR' # 10, Fig. 9h 5 and j), a dissociation between plasma inhibin and plasma oestradiol concentrations was observed, suggesting different regulation of these two parameters of follicular development. As inhibin is a peptide produced by ovarian granulosa cells and plasma oestradiol in the human is predominantly an ovarian 10 theca cell product, the assay of plasma inhibin is the first direct plasma parameter of granulosa cell/oocyte health and maturation. The dissociation of plasma inhibin and  $E_2$  may therefore be of therapeutic importance in that the plasma inhibin is a direct measure of follicular development, and its 15 assessment may affect the timing of ovulation induction and oocyte collection.

Example 12    hFF inhibin as standard for radioimmunoassay

Human follicular fluid (hFF) obtained at oocyte collection in the IVF programme was prepared for use as the 20 radioimmunoassay (RIA) standard by two gel chromatographic steps and reversed phase HPLC as described for bFF inhibin (Robertson et al, 1985). This material yielded parallel dose response lines to human female serum inhibin obtained from women undergoing ovarian hyperstimulation for in vitro 25 fertilisation. This partially purified human follicular fluid (hFF) inhibin standard preparation was defined in terms of its in vitro inhibin bioactivity using an inhibin bioassay based on the dose-related suppression of FSH cell content (Scott et al, 1980). This material was used as the RIA standard and 30 gave dose response lines parallel to serum inhibin obtained from women undergoing ovarian hyperstimulation for in vitro fertilization and also to that of pregnant serum.

The unitage of the hFF inhibin standard was calibrated in terms of an ovine testicular lymph standard 35 preparation of defined unitage 1 U/mg using the inhibin

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bioassay. The RIA has an interassay coefficient of variation (CV) of 6.4% (n = 5 assay) and the sensitivity (logit + 2) was 0.37 U/ml.

The RIA was specific to bovine and human inhibin and cross-reacted less than 0.3% with a range of glycoproteins and growth factors. In addition, inhibin-related peptides cross-reacted as follows: porcine transforming growth factor  $\beta$  < 0.9%, bovine Mullerian Inhibitory Substance 0.3%, purified bovine inhibin B subunit dimer <1% and the subunits of 31kDa bFF inhibin following reduction and alkylation <0.1%. No immunoactivity was detectable in the sera of castrate subjects, post-menopausal women, nor in a subject with Turner's syndrome. The RIA had an interassay coefficient of variation of 8.3% (n = 5 assays), and a sensitivity of 0.37 U/ml.

Inhibin levels were determined at 1 dilution level against the partially purified hFF standard preparation using an iterative curve-fitting procedure (Burger *et al.*, 1973). In the calculation of results, a lognormal distribution of individual observations (Gaddum *et al.*; 1933) was assumed, i.e. all calculations were performed using logarithmically transformed values to give geometric means and 67% confidence intervals. Statistical comparison between pregnant and non-pregnant groups was performed using the unpaired t-test.

25 Example 13      Inhibin levels during luteal phase and early pregnancy

Nineteen women presenting consecutively for treatment in the Mondsh university IVF program were studied. Clinically their infertility resulted from tubal disease (n = 30 7), endometriosis (n = 6), unknown causes (n = 5) or poor semen quality (n = 4). The protocol of ovulation induction has been described elsewhere (Wood and Trounson, 1984). Briefly, all subjects received clomiphene citrate (Clomid, Merrell Dow, Sydney) 100-150 mg daily between days 5 and 9 of the cycle and 35 HMG (Pergonal, Serono, Rome) 75-225 units daily from day 6. The dosage and duration of HMG therapy were optimized

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according to daily plasma oestradiol concentrations and follicular size as assessed by ovarian ultrasound. HCG (Pregnyl, Organon, Oss) 5,000 IU intramuscularly was administered to induce ovulation, and oocyte retrieval was undertaken 36 hours later. Embryo transfer was performed as described by Wood and Trounson (1984). Blood was taken on day 1 post laparoscopy and every second day from day 2 to day 14 and sera stored for measurement of FSH, LH,  $\beta$  subunit hCG, oestradiol, progesterone and inhibin. Three of the 19 women became pregnant.

At various stages of gestation, a single serum sample was obtained from each of 24 normal pregnant women.

Samples were assayed for inhibin using the hFF inhibin standard described in Example 11. In the 16 patients who did not conceive, luteal phase inhibin levels rose to a peak level of 2.5 U/ml on day 6 and then fell to undetectable levels by day 14. These results are shown in Figure 13.

The number of subject serum samples per day was 13-16 except at day one when only eight were available. Results are expressed as the geometric mean  $\pm$  67% confidence intervals. The broken line indicates the limit of sensitivity of the inhibin radioimmunoassay. The number of subjects showing non-detectable inhibin values is shown in parentheses. Non-detectable values are not included in the mean  $\pm$  confidence intervals.

In three subjects who conceived, inhibin levels were similar to non-conception cycles between days 2 and 8, increasing thereafter and becoming significantly higher ( $p < 0.001$ ) than in the non-pregnant group by day 12 post-laparoscopy. Figure 14 shows these results, expressed as the geometric mean  $\pm$  67% confidence intervals. The broken line indicates the sensitivity of the inhibin radioimmunoassay.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  comparing hormone values for the pregnant and non-pregnant groups on the same day. Significance values in the second panel refer to serum FSH. The late luteal phase rise in serum inhibin in the

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pregnant patients coincided with both the rise in serum  $\Delta$ hCG and with the decline in serum FSH to values below those seen in the non-pregnant group.

Serum FSH showed a significant inverse correlation with inhibin in the luteal phase of the non-conception cycles ( $r = 0.51$ ,  $n = 113$ ,  $p < 0.001$ ) (Fig. 15). Similar significant inverse relationships were observed between FSH and inhibin when the data were analysed according to whether the progesterone concentrations were in the normal ovulatory luteal phase range (25-100) nm or greater, ( $r = 0.38$ ,  $n = 76$ ,  $p < 0.001$  vs  $r = 0.37$ ,  $n = 37$ ,  $p < 0.05$ , slopes not statistically different). Significant inverse correlations also existed between luteal phase serum FSH and both progesterone ( $r = 0.64$ ,  $n = 113$ ,  $p < 0.001$ ) and oestradiol ( $r = 0.52$ ,  $n = 114$ ,  $p < 0.001$ ).

Plasma inhibin and progesterone concentrations were significantly correlated in the luteal phase of cycles in which pregnancy did not occur ( $r = 0.81$ ,  $n = 85$ ,  $p < 0.001$ ), as were plasma inhibin and oestradiol concentrations ( $r = 0.65$ ,  $n = 85$ ,  $p < 0.001$ ). In pregnant subjects, luteal phase inhibin levels did not show significant correlations with either progesterone or oestradiol. Serum LH levels (not shown) fell sharply from day 1 (21.0 [17.0-26.1] mIU/ml) to a nadir (3.5 [1.2-9.8] mIU/ml) on day 8.

In a separate study of serum inhibin, levels were determined during gestation in 24 normal pregnant women. The mean level prior to 20 weeks gestation (1.31 (0.95-1.80) U/ml,  $n = 13$ ) was significantly lower ( $p < 0.02$ ) than levels after this time (2.02 U/ml (1.32-3.10) U/ml,  $n = 11$ ).

Thus there is a rise in circulating inhibin concentrations during the luteal phase of stimulated menstrual cycles and during pregnancy.

Example 4      Inhibin Levels in the Normal Menstrual Cycle

In a further study, serum inhibin was determined daily in 6 normal women throughout the menstrual cycle, using a radioimmunoassay employing an antiserum directed to 31kD inhibin. The normalcy of the menstrual cycle was assessed from the serum profiles of FSH, LH, progesterone, and oestradiol. The increase in sensitivity of the assay using this antiserum permitted the detection of inhibin in over 97% of samples. The results are shown in Fig. 16.



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10 Advantages and applications of the assay according to the invention.

1. The assay may be used for determining inhibin concentration in a wide range of biological samples, such as serum, plasma, urine, follicular fluid, tissue homogenates, and culture fluids.
2. The assay may be used to monitor the purification of inhibin from tissue, biological fluids, or culture medium, or to monitor transfection studies.
3. Inhibin levels may be used as a marker of parameters of reproductive function, such as granulosa cell function, follicular development, number of ovarian follicles following ovarian hyperstimulation, and foetal well-being during early pregnancy, and Sertoli cell function.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

The following terms referred to hereinbefore are trade marks: Amerlex-M, Clomid, Coat-a-Count, Marcol 5 2, Matrex Red A, Montanide 888, Norit A, Pergonal, Polypep, Pregnyl, RIA-Quant, Sephacryl, Sephadex, Triton X-100, Ultrapore, and Ultra-Turrax.

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## CLAIMS:

1. A method of immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin.
2. A method according to claim 1 in which the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof.
3. A method according to claim 2 in which the antigen is selected from the group consisting of preparations containing inhibin, purified bovine 58kD inhibin, purified bovine 31kD inhibin, human inhibin, or human or bovine inhibin or sub-units, fragments or derivatives thereof produced using recombinant DNA technology.
4. A method according to claim 1 in which the antibody is a monoclonal antibody.
5. A method according to claim 1 in which the antibody is capable of neutralizing inhibin bioactivity.
6. A method according to claim 1 which further comprises the step of using labelled 58kD or 31kD inhibin as tracer.
7. A method according to claim 1 in which the assay is a radioimmunoassay, an enzyme-linked immunosorbent assay, or an immunoassay based on fluorescence detection.
8. A method according to claim 1 in which an assay standard is used, said assay standard being selected from the group consisting of naturally-occurring or recombinant inhibin, or sub-units, fragments or derivatives thereof.
9. A method according to claim 8 in which the standard displays parallelism in the assay with the samples under test.

10. A method according to claim 8 in which the standard is selected from the group consisting of bovine 31kD inhibin, partially purified human inhibin, and purified human inhibin.
11. A radioimmunoassay for measuring inhibin in a biological sample, comprising the steps of:
- a) incubating sample and antiserum for 4 hours to 4 days at 4° to 30°C,
  - b) adding  $^{125}\text{I}$ -inhibin and incubating either overnight at room temperature, for 48 to 72 hours at 4°C, or for 16 hours at 30°C,
  - c) Adding a second antibody and incubating for 30 min. to 24 hours at 4°C,
  - d) Separating precipitate, and
  - e) counting bound  $^{125}\text{I}$ -labelled inhibin.
12. A radioimmunoassay according to claim 11 in which samples to be assayed are diluted in inhibin-free serum.
13. A radioimmunoassay according to claim 11 in which an assay standard is used, said standard being selected from the group consisting of naturally-occurring or recombinant bovine 31kD inhibin and naturally-occurring or recombinant human inhibin.
14. A radioimmunoassay according to claim 11 in which incubation with  $^{125}\text{I}$ -inhibin is at 30°C.
15. A radioimmunoassay according to claim 11 in which polyethylene glycol is added following incubation with the second antibody and incubated for a further 30 minutes.
16. A radioimmunoassay according to claim 11 in which Triton X-100 is incorporated into samples to be assayed.
17. A method for measuring inhibin in samples such as follicular fluid or serum from various species (including humans) wherein concentrations of inhibin in standards are used to derive the concentration of inhibin in the follicular

fluid or serum by competitive binding of  $^{125}\text{I}$ -labelled inhibin and inhibin from test samples with bovine 58kD inhibin antiserum, followed by precipitation and counting of bound  $^{125}\text{I}$ -labelled inhibin.

18. A method for preparation and purification of  $^{125}\text{I}$ -labelled inhibin tracer which comprises the steps of iodination of inhibin using a Chloramine T procedure and purification of  $^{125}\text{I}$ -inhibin by an affinity fractionation step.

19. A method according to claim 18 in which the affinity fractionation step uses Matrex Red A.

20. A method according to claim 18 which additionally comprises a gel filtration step.

21. An assay standard for estimation of inhibin according to the method defined in claim 1, claim 11 or claim 17, selected from the group consisting of naturally-occurring or recombinant inhibin, or fragments or derivatives thereof.

22. An assay standard according to claim 21 selected from 31kD bovine inhibin and human inhibin.

23. A test kit for the estimation of inhibin in a sample, comprising an agent selected from the group consisting of:

- a) labelled inhibin,
- b) antibody directed against inhibin,
- c) an assay standard as defined in claim 21.

24. A test kit according to claim 23 in which labelled inhibin is prepared according to the method of claim 18.

25. A test kit according to claim 23 in which the antibody to inhibin is contained in an antiserum as defined in claim 2.

26. Products and processes substantially as hereinbefore described with reference to the accompanying drawings.

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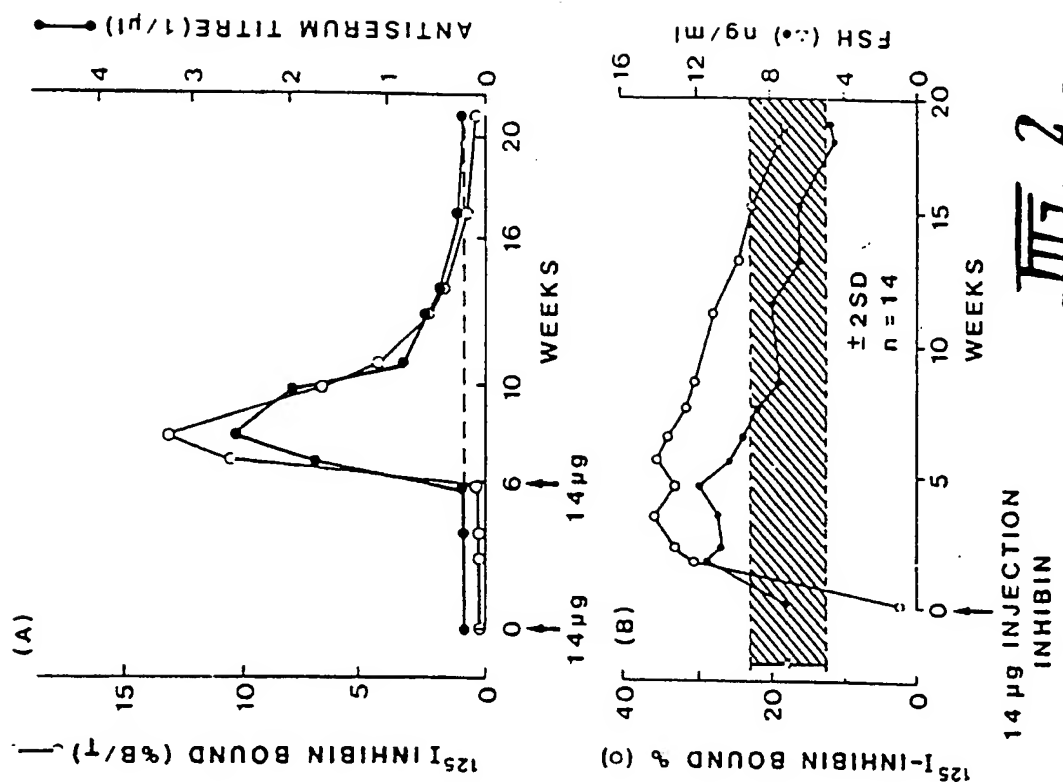


Fig. 2.

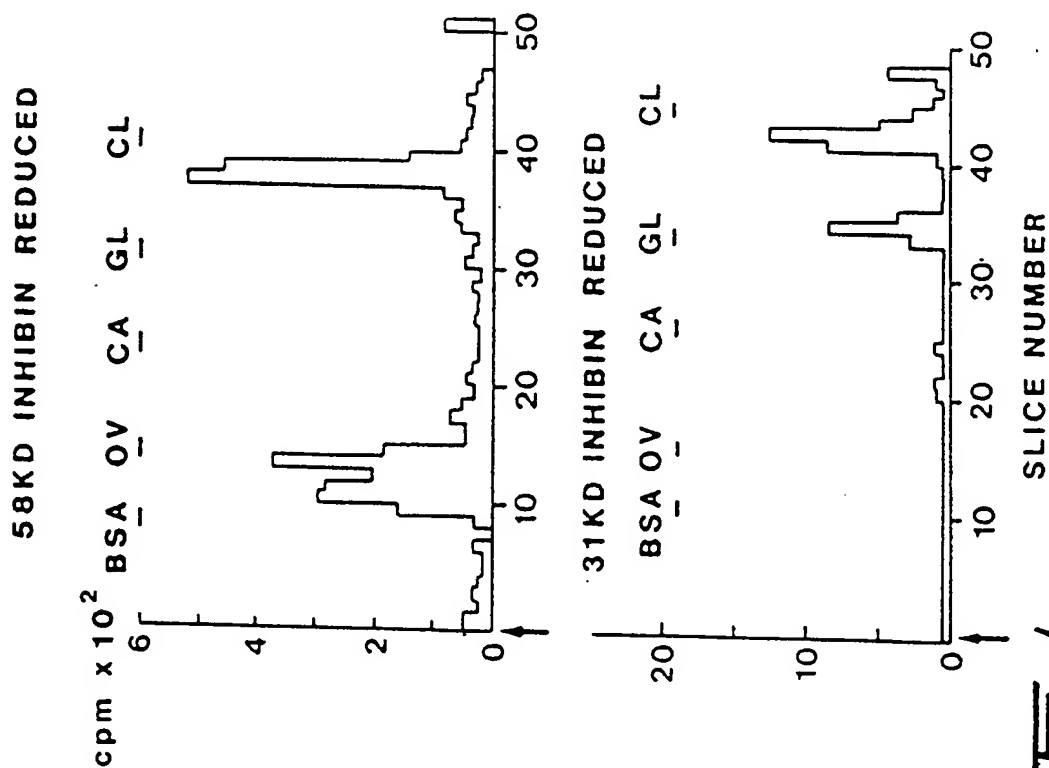
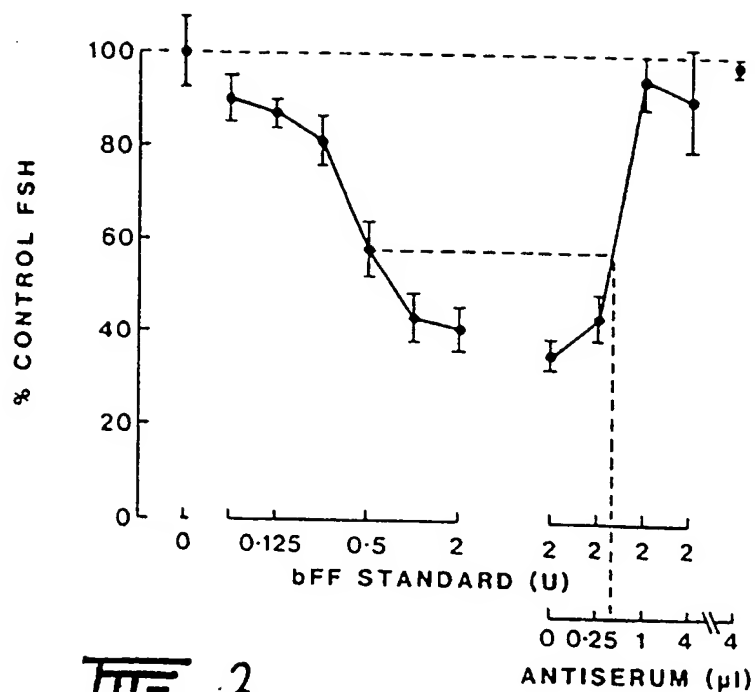
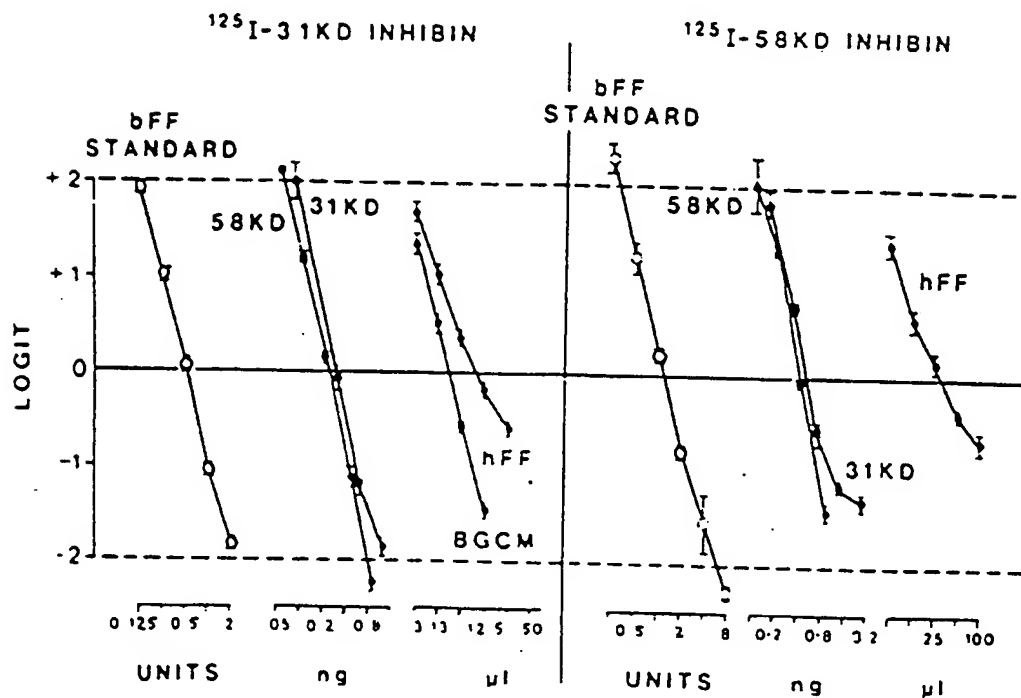


Fig. 1.

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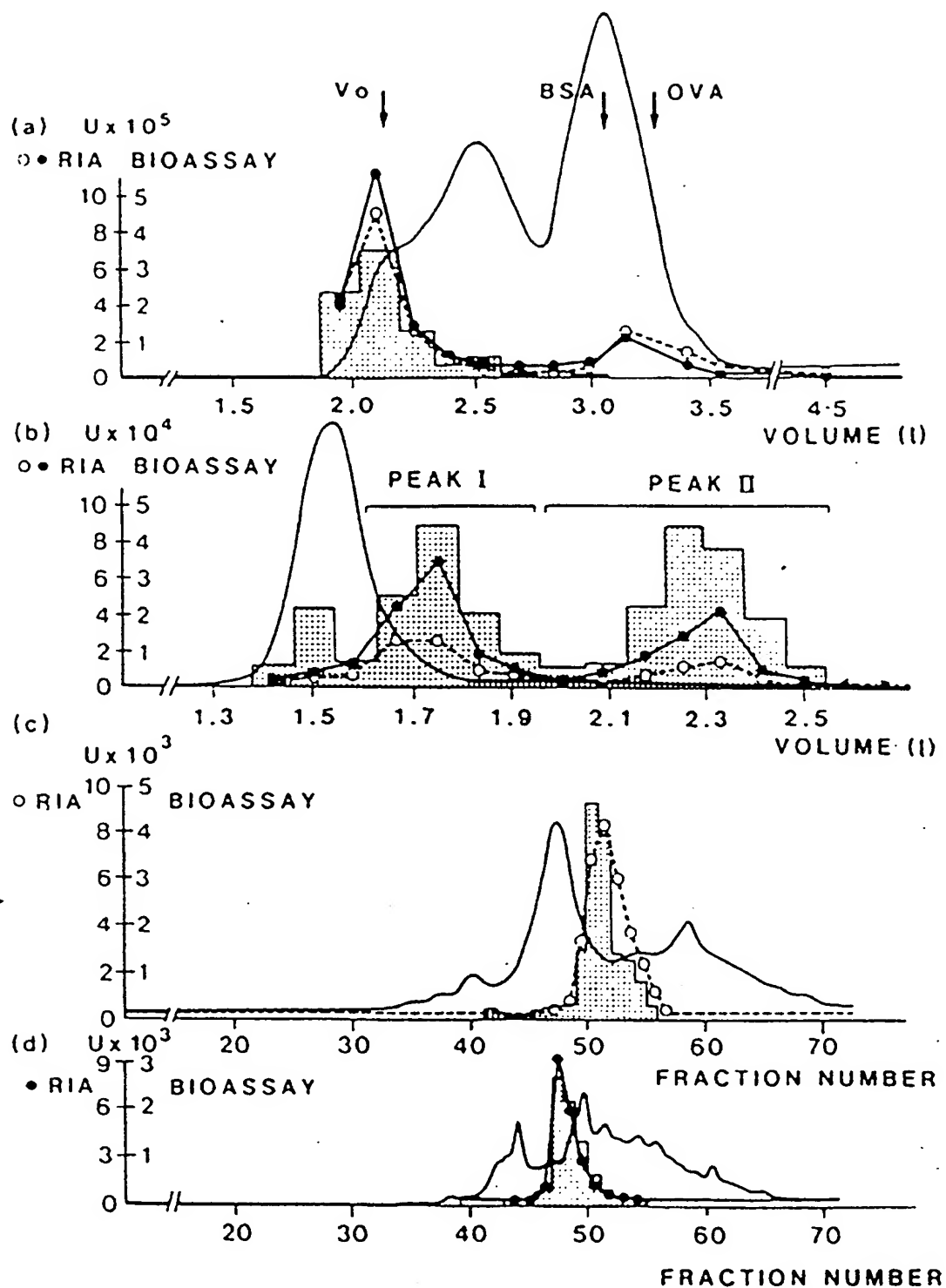
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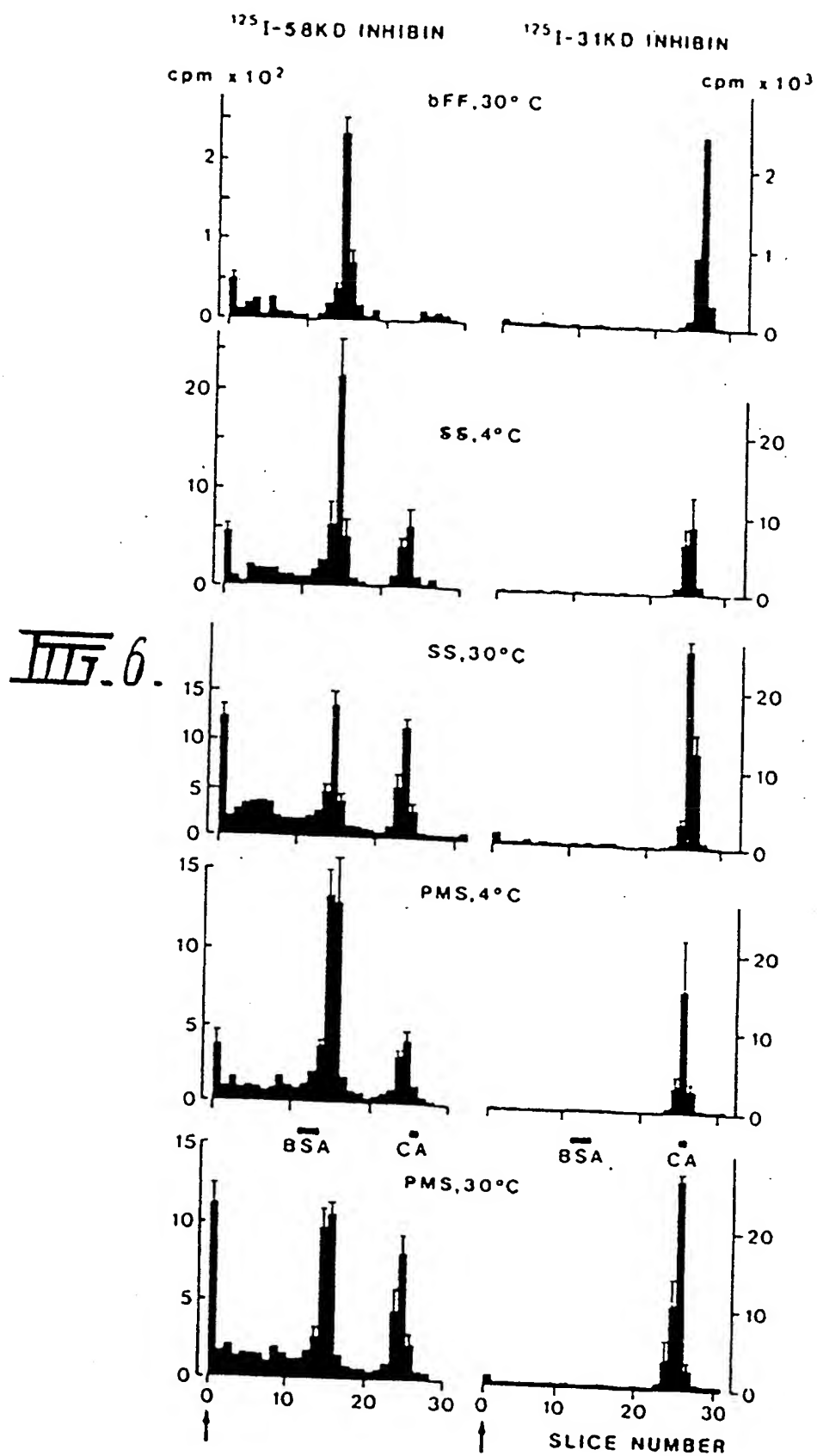
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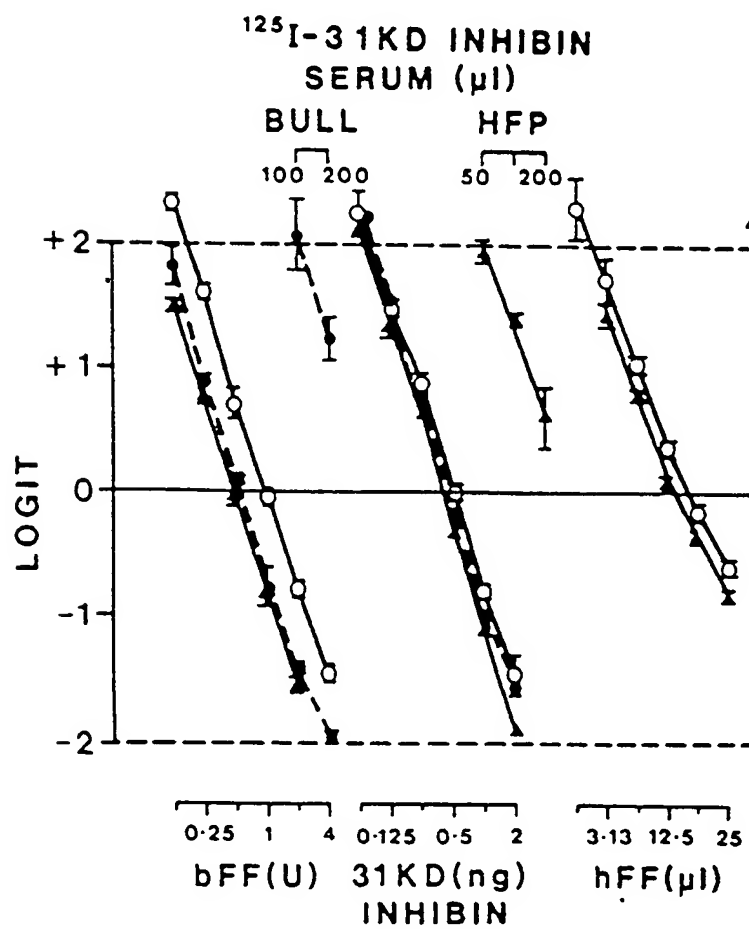
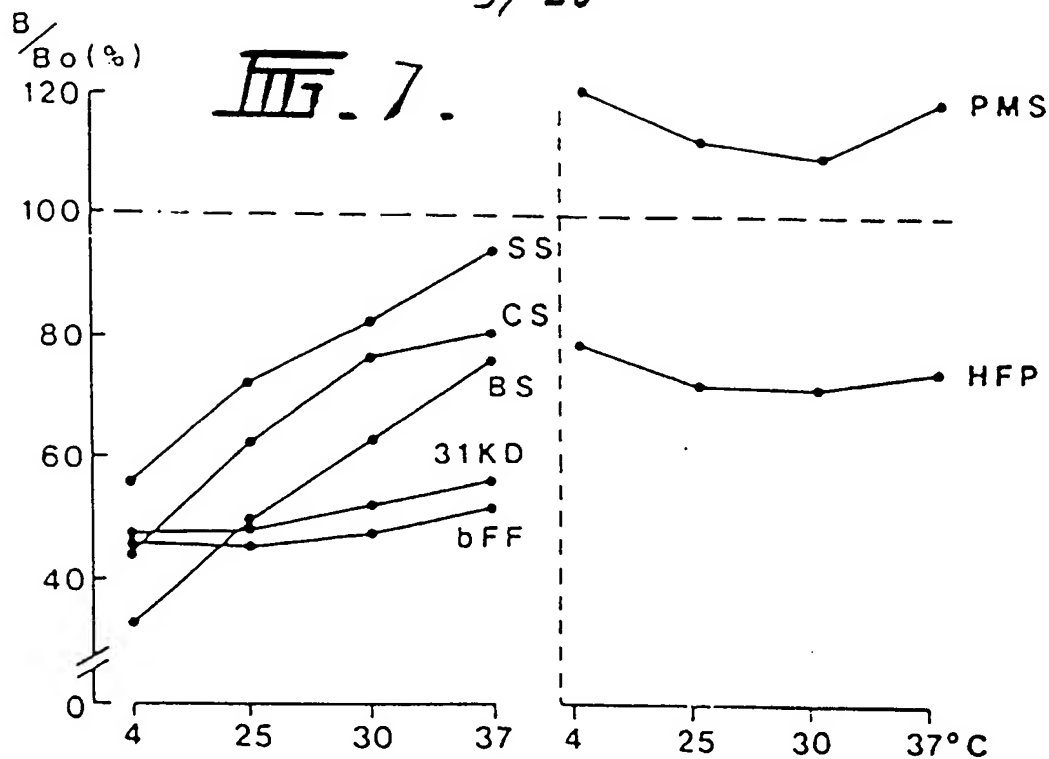
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Fig. 5.

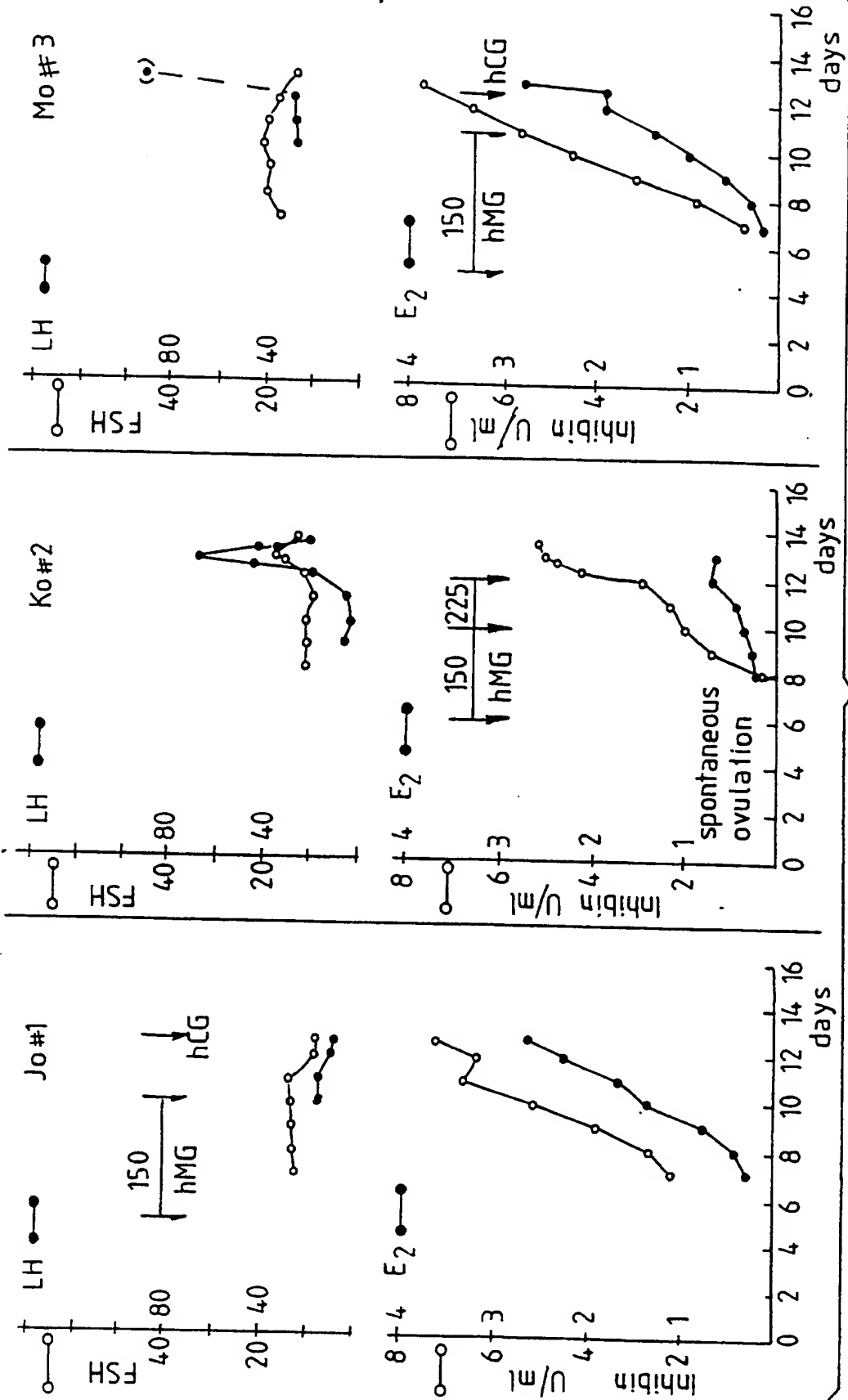
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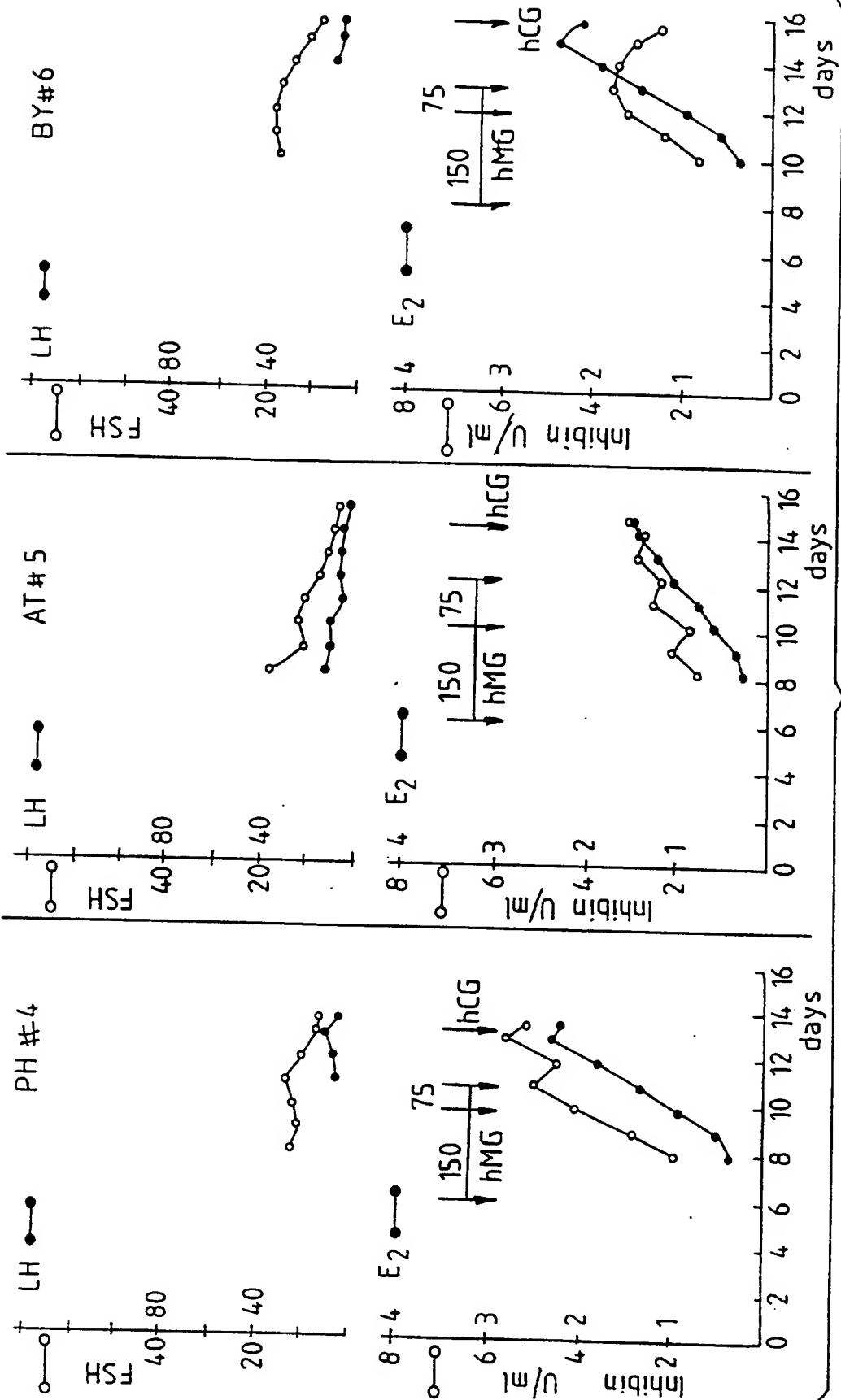
(c)

(b)

(a)

Fig. 9

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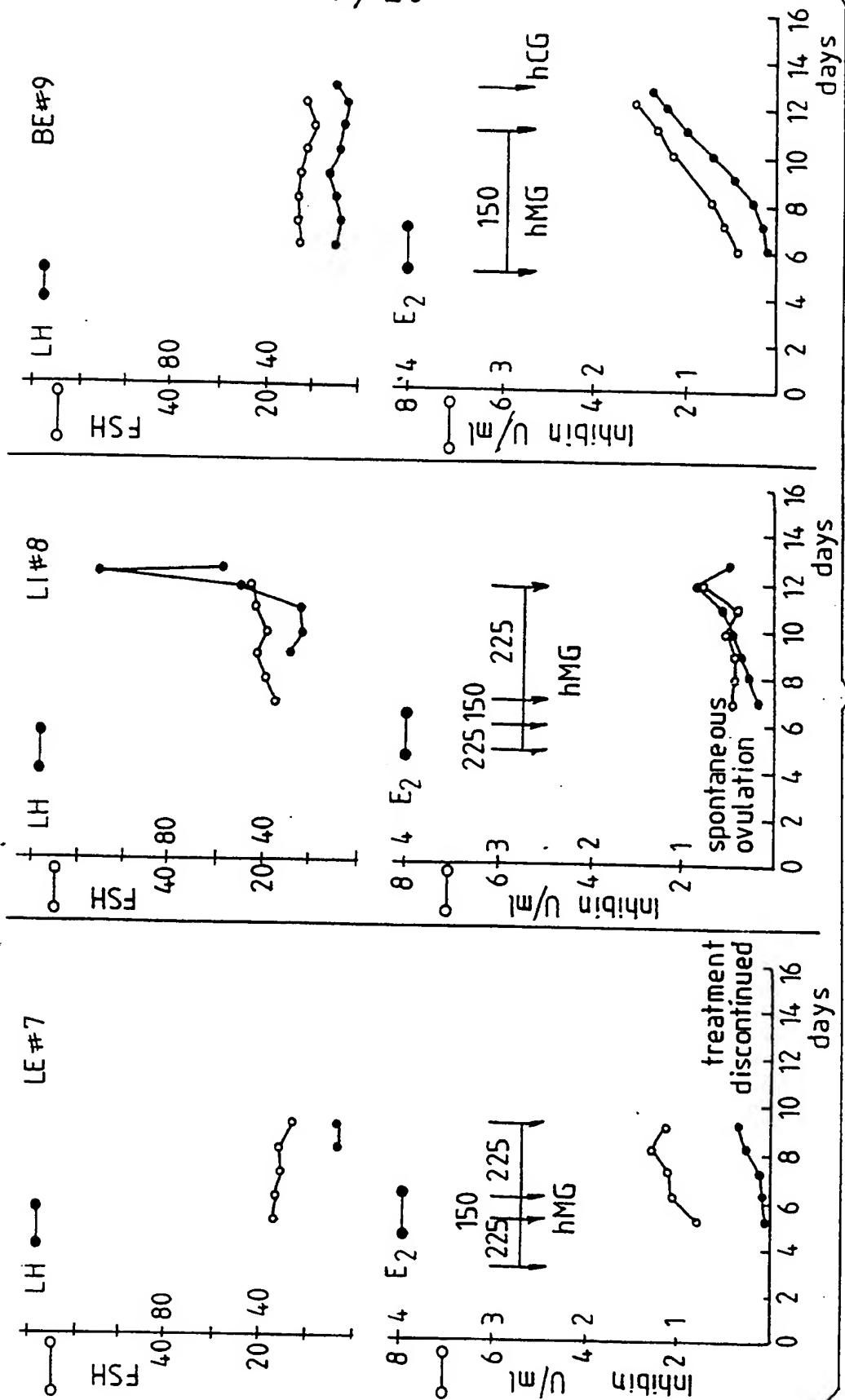


(f)

III. 9. (e)

(d)

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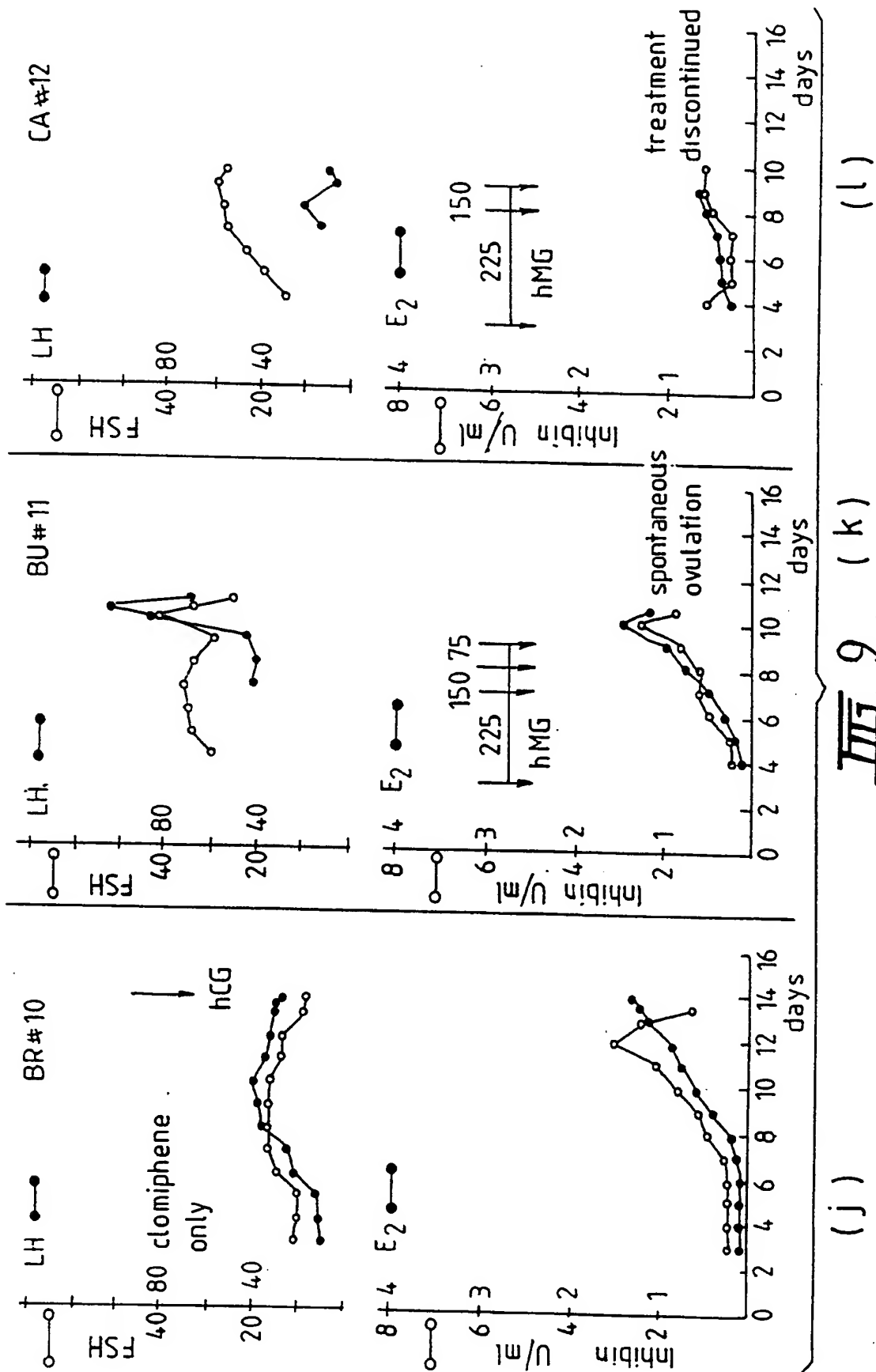


(i)

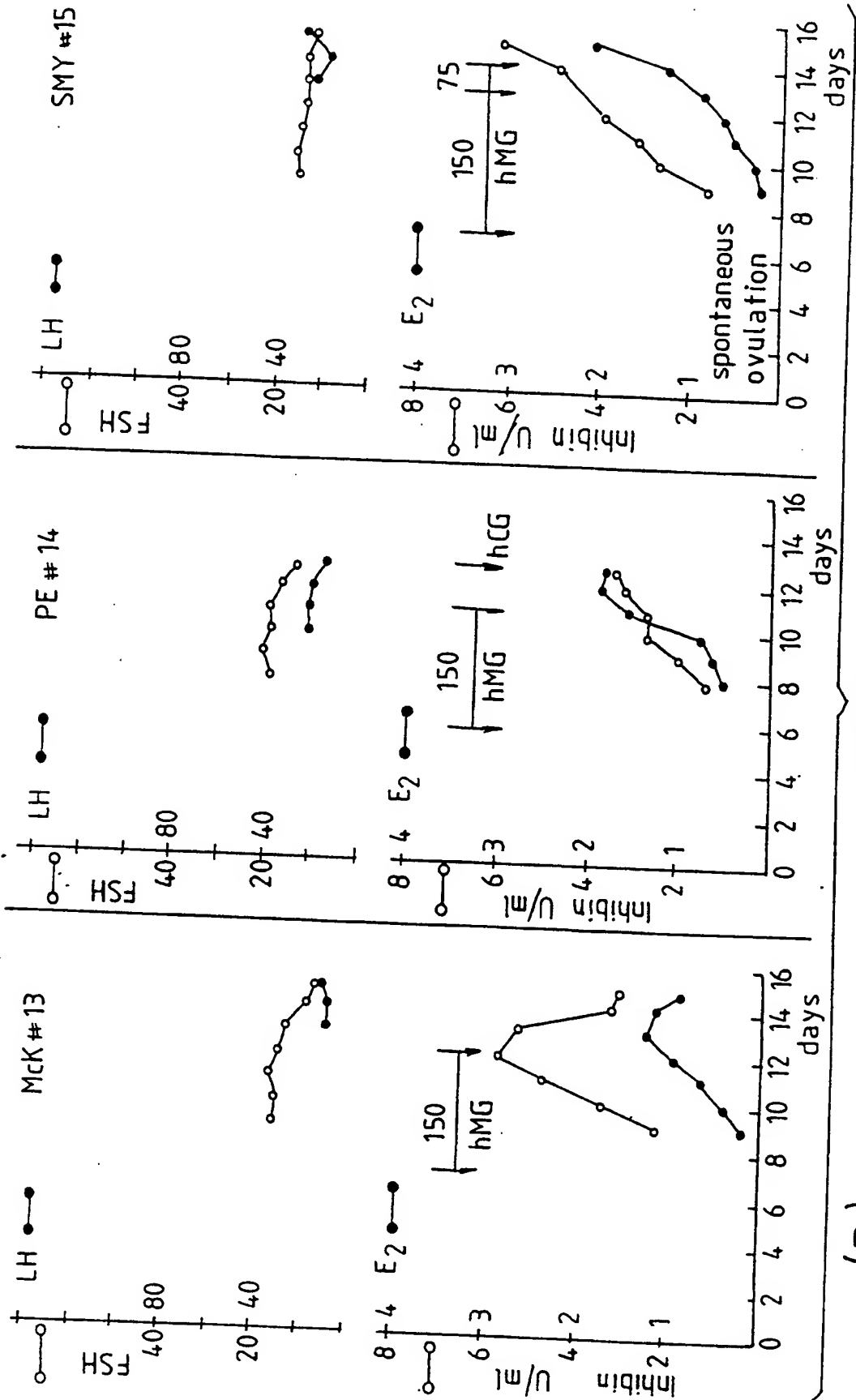
Fig. 9 (h)

(g)

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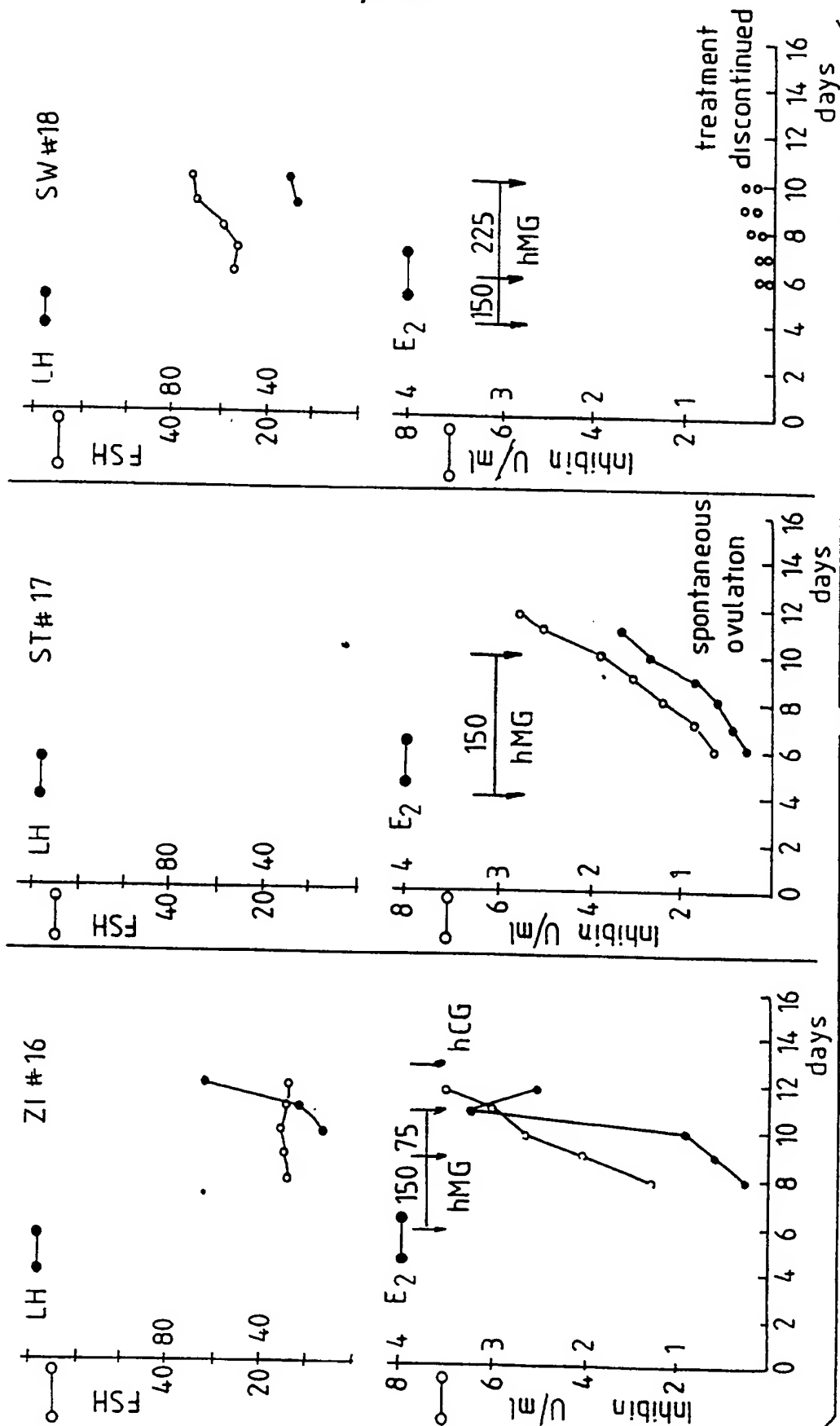
(o)

III. 9. (n)

(m)



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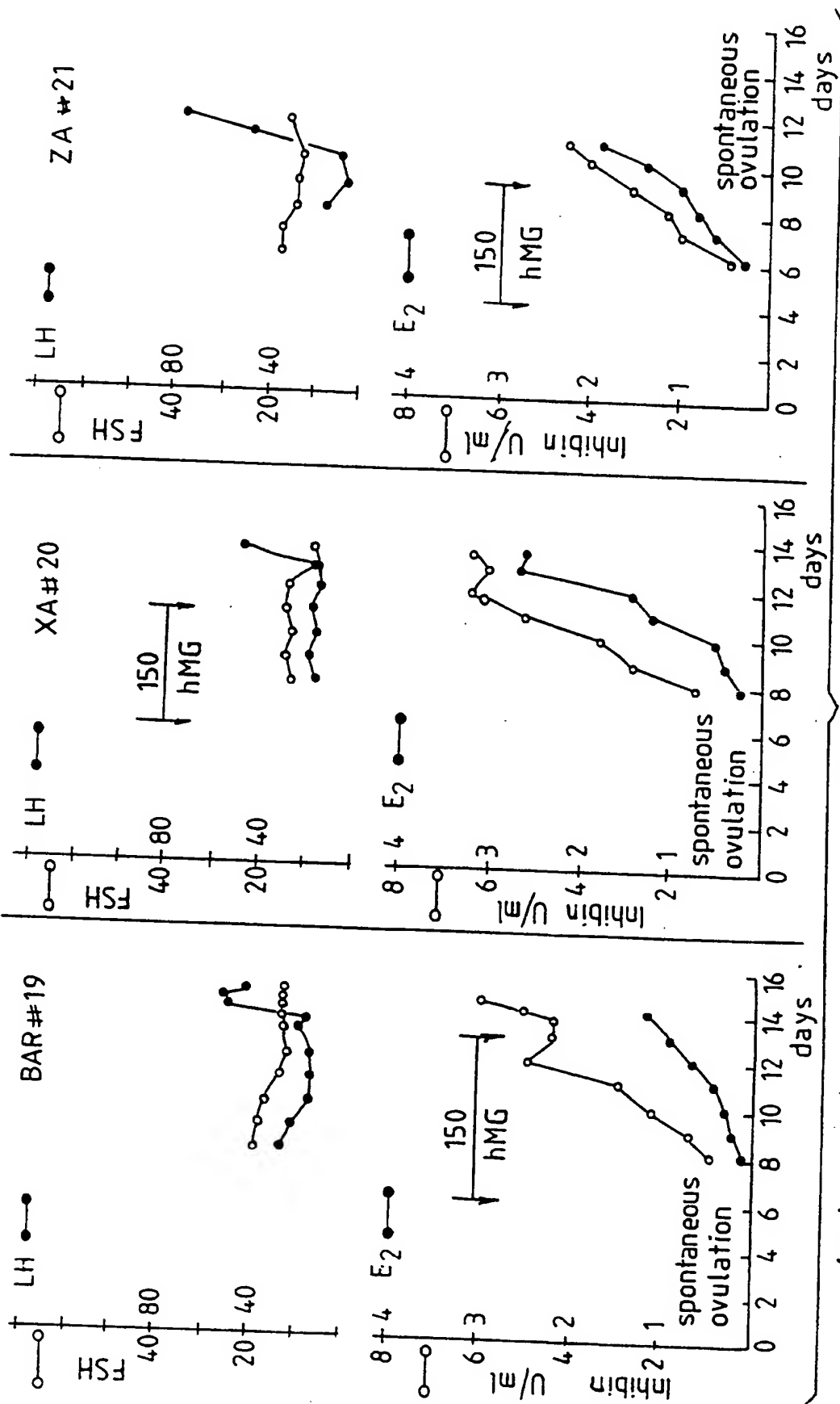


(p)

MG-9 (q)

(r)

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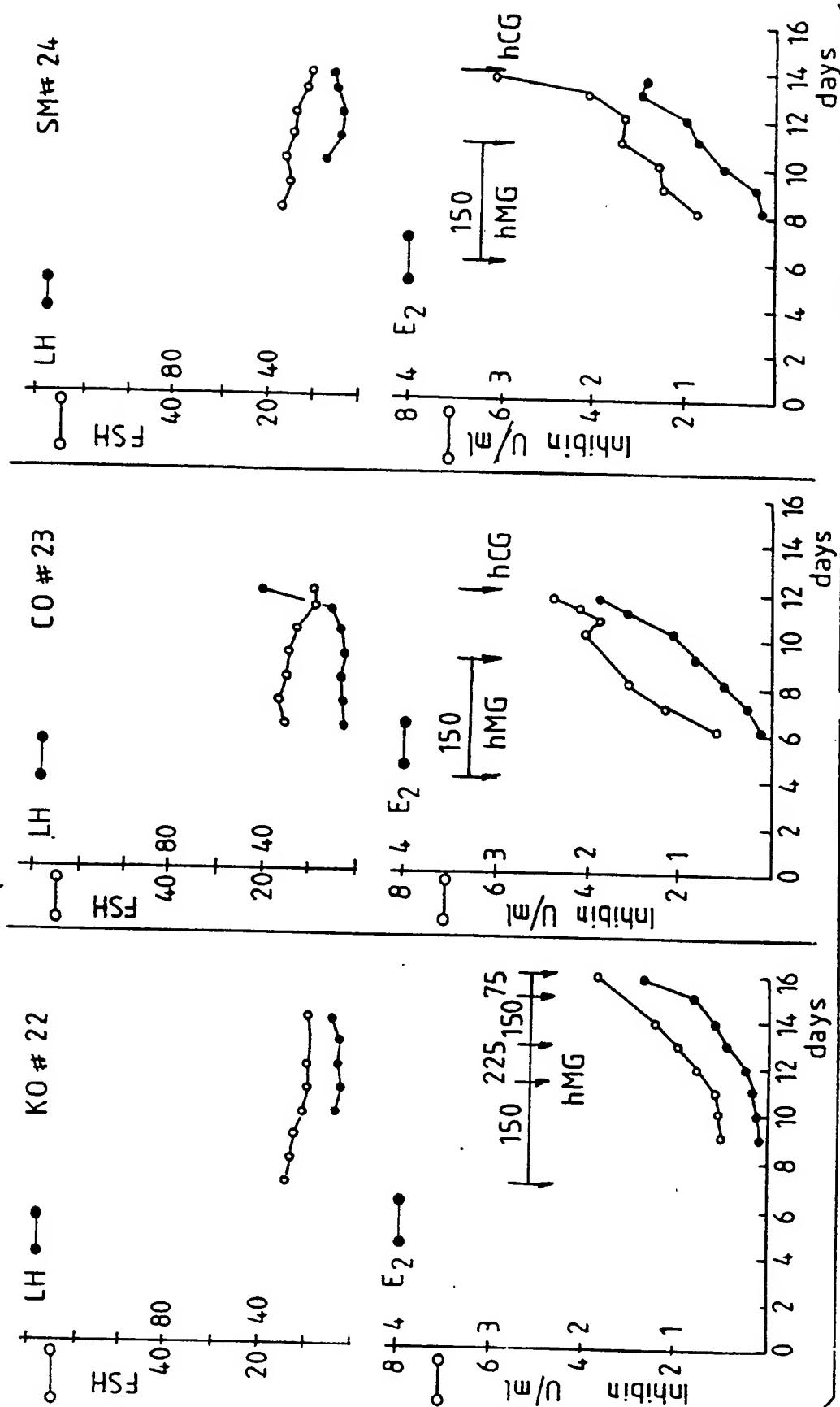


(u)

Fig. 9 - (t)

(s)

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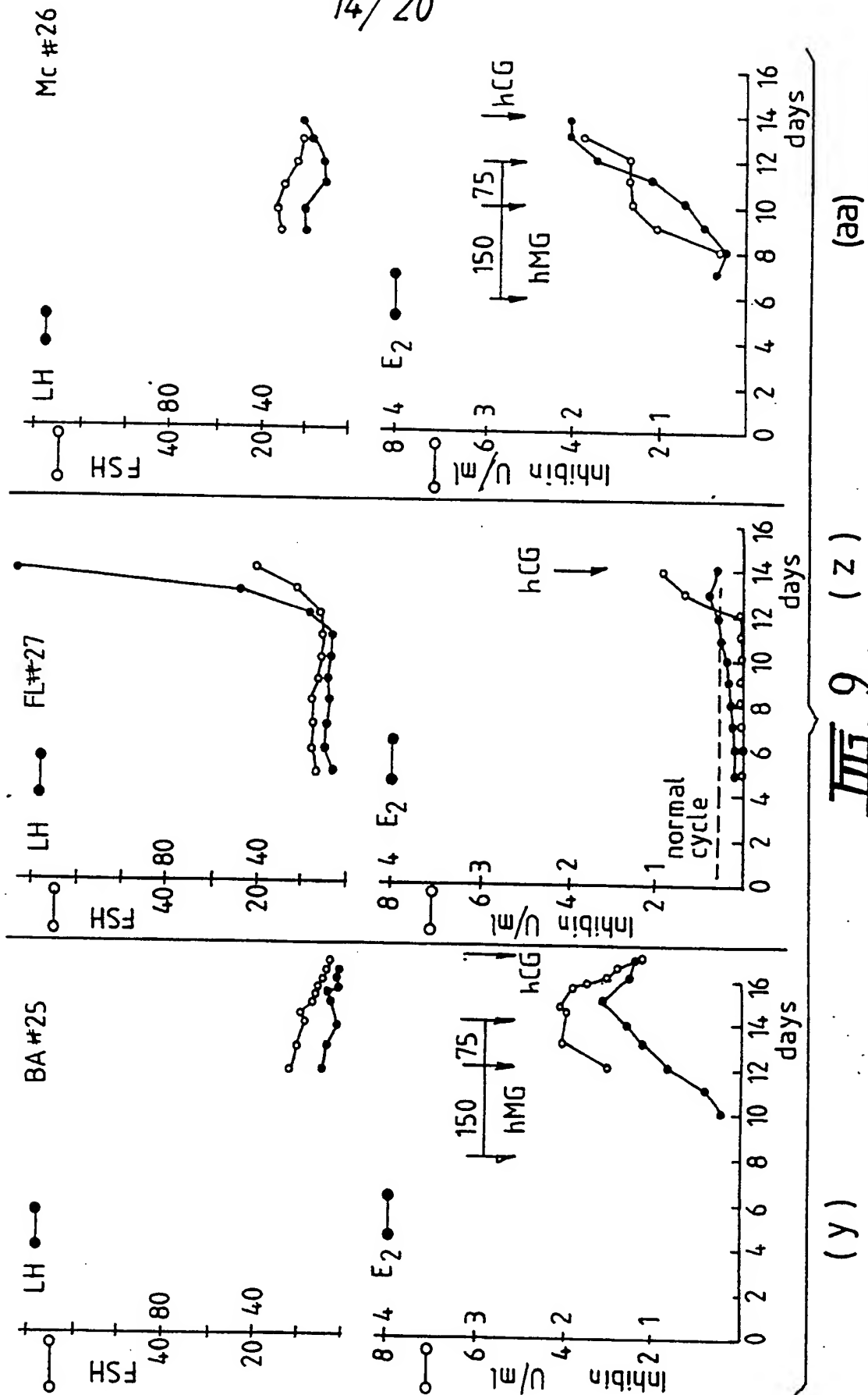
(x)

(w)

(v)

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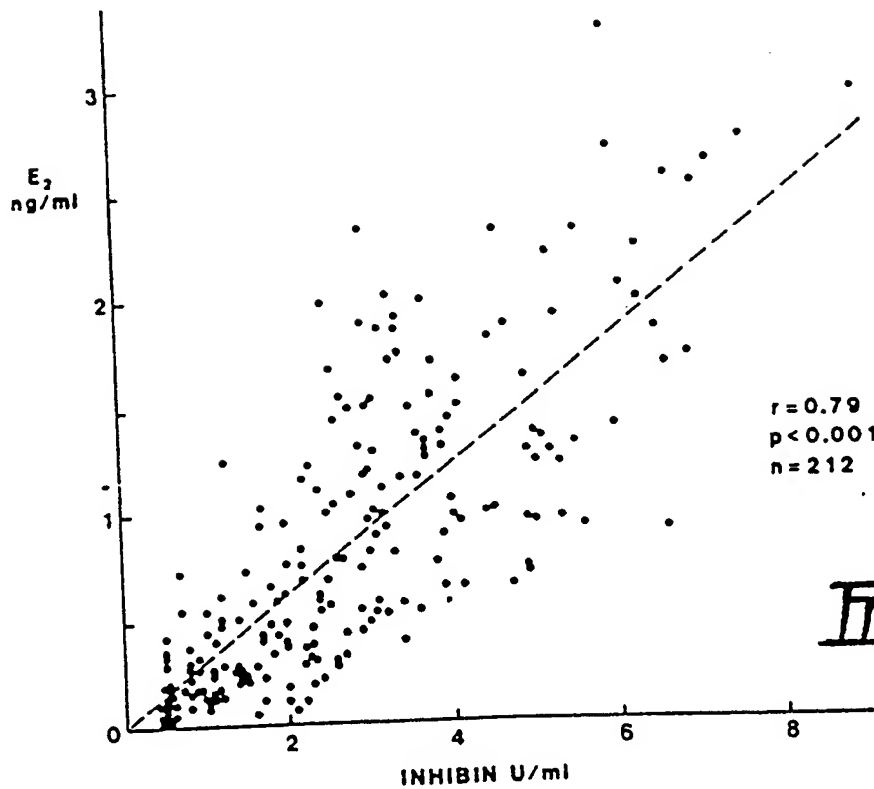


FIG. 10.

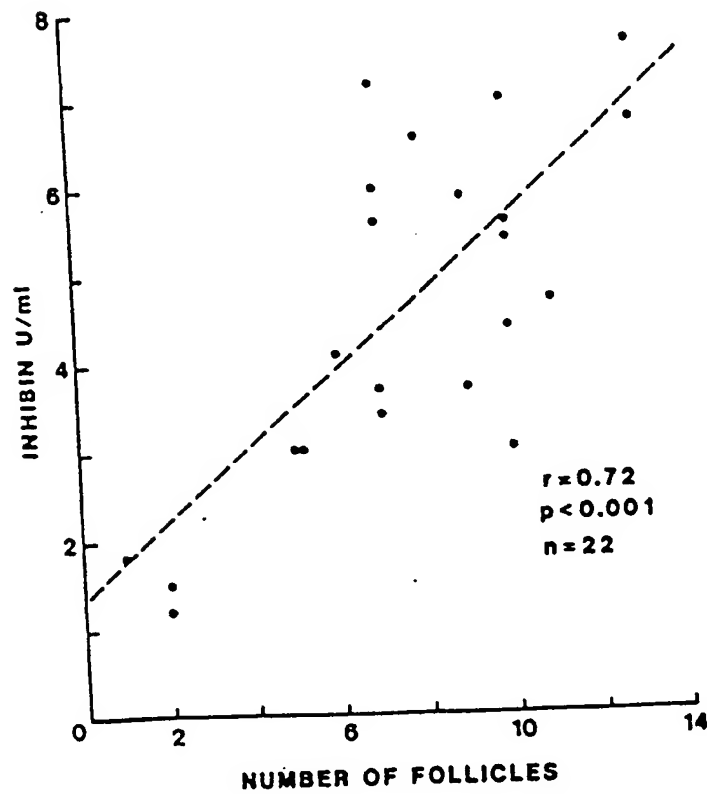
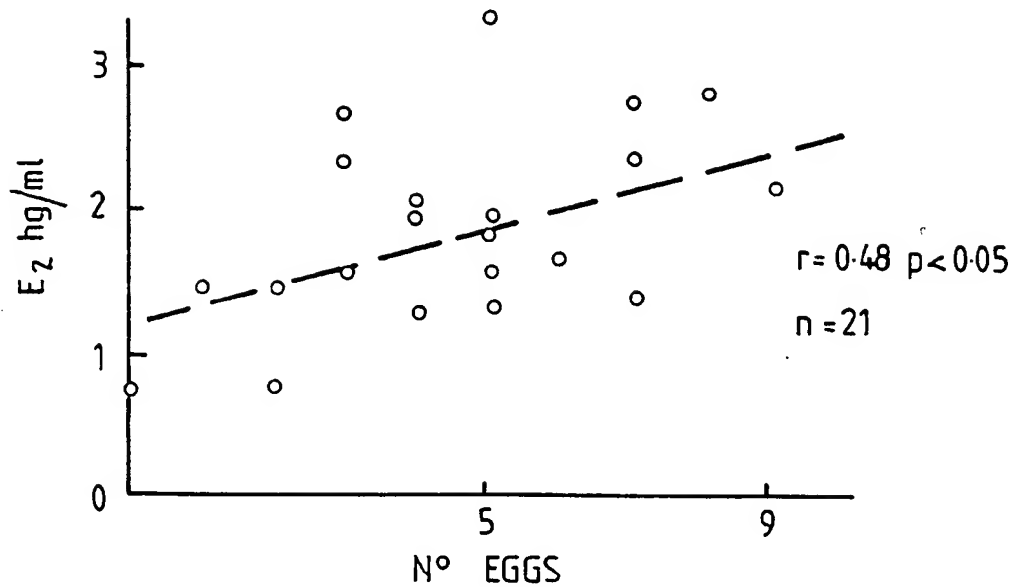


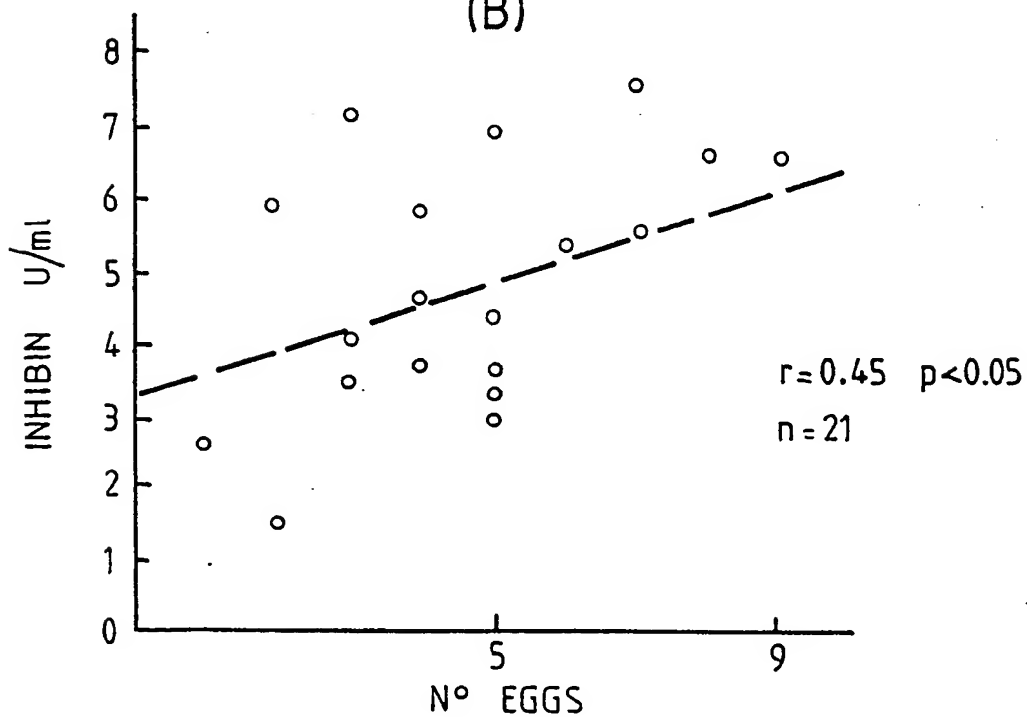
FIG. 12.

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(A)

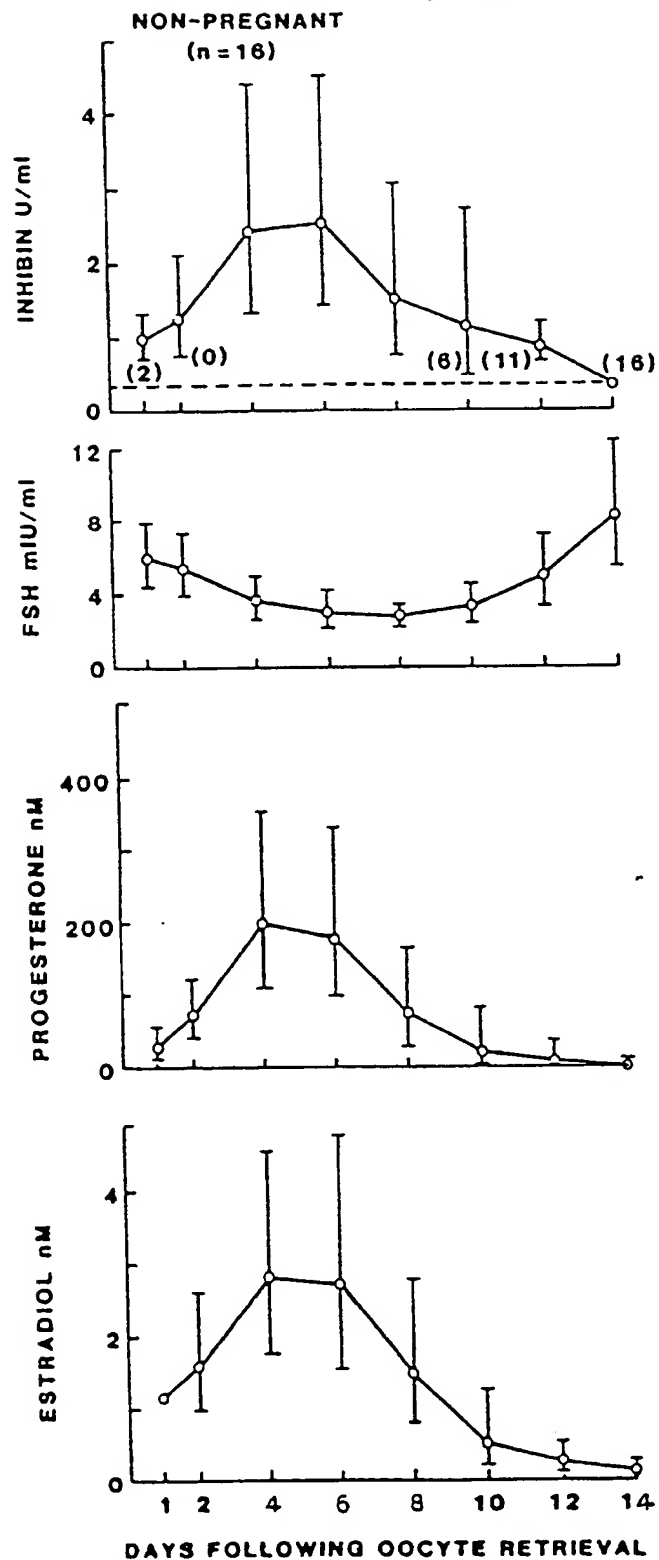


(B)

Fig. 11

Comparison of eggs recovered at  
Laparoscopy and Peak Plasma levels  
of  $E_2$  and INHIBIN

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III. 13.

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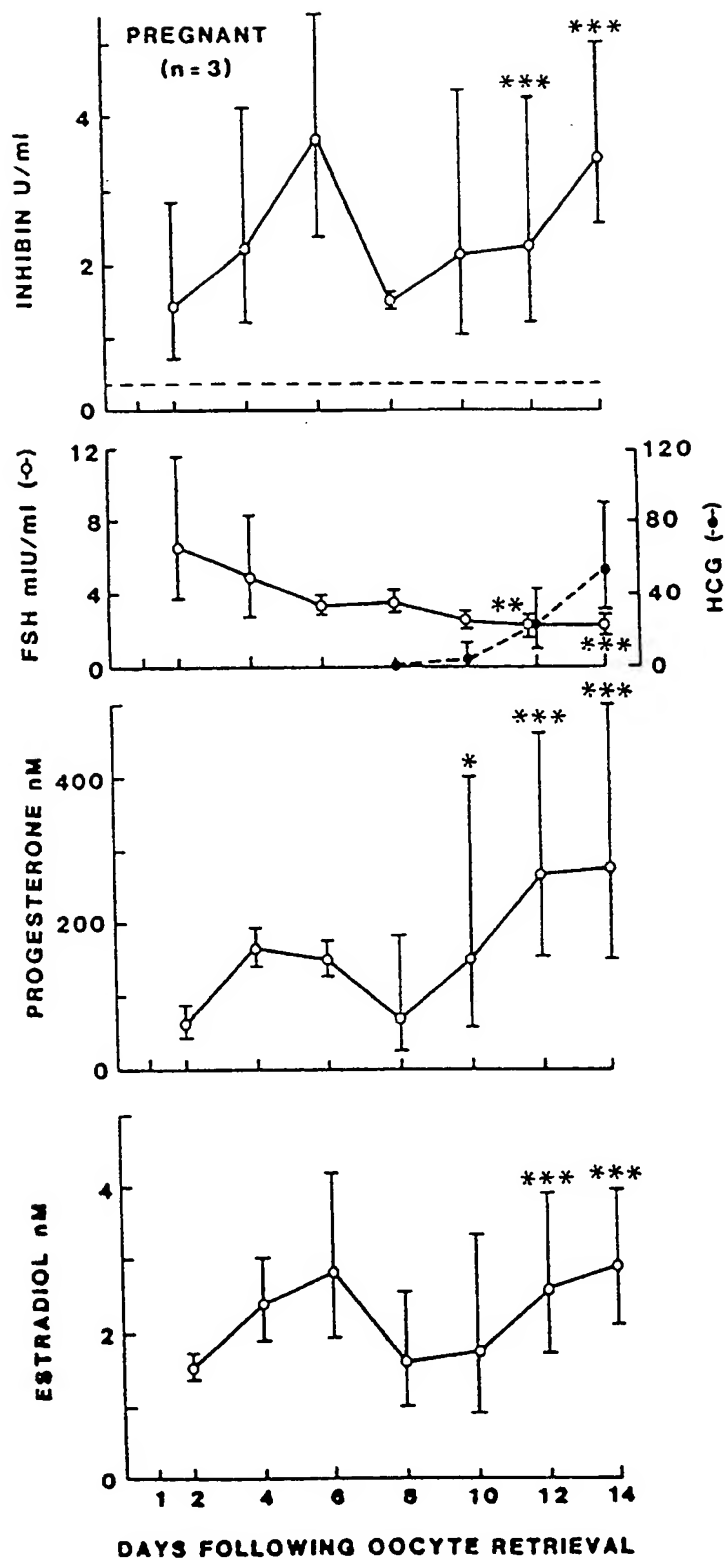
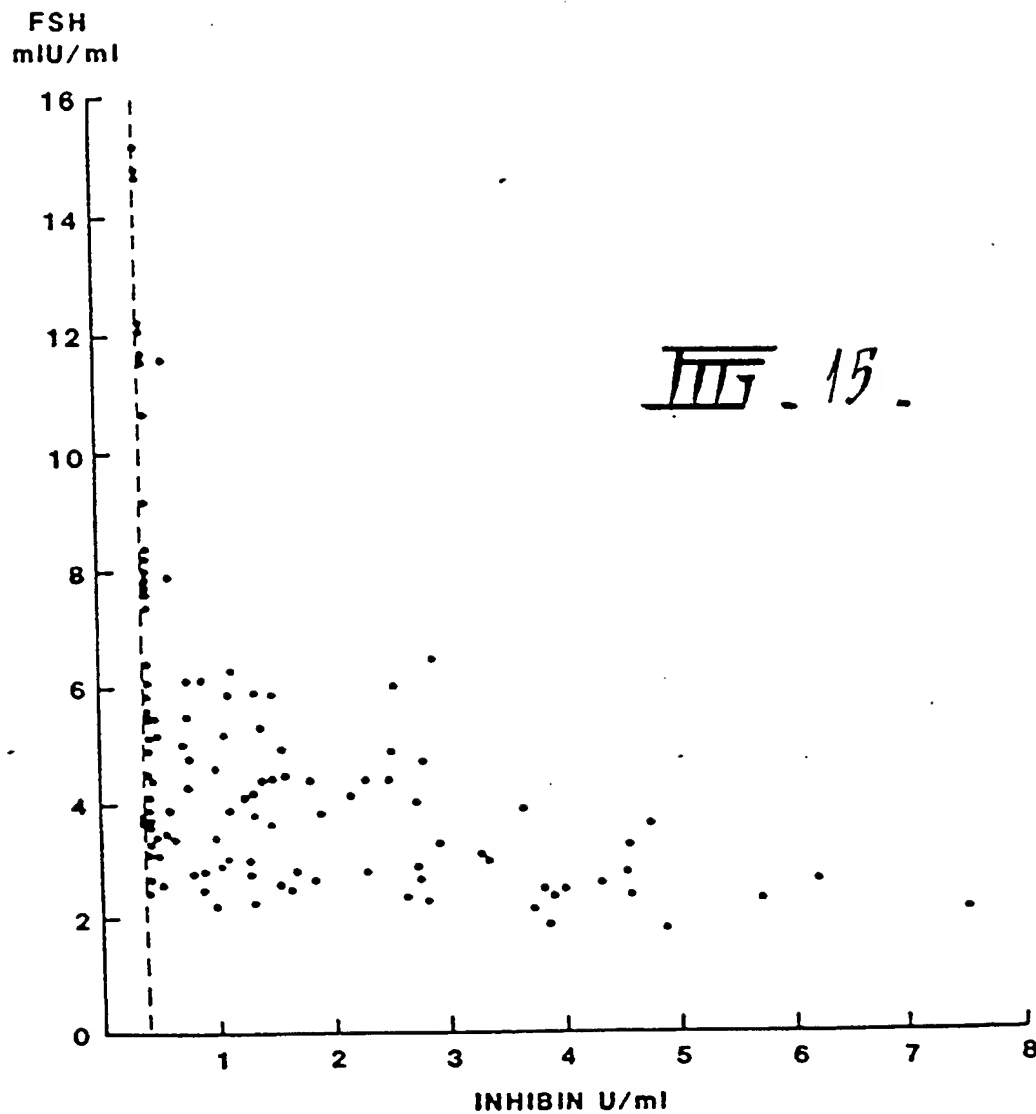


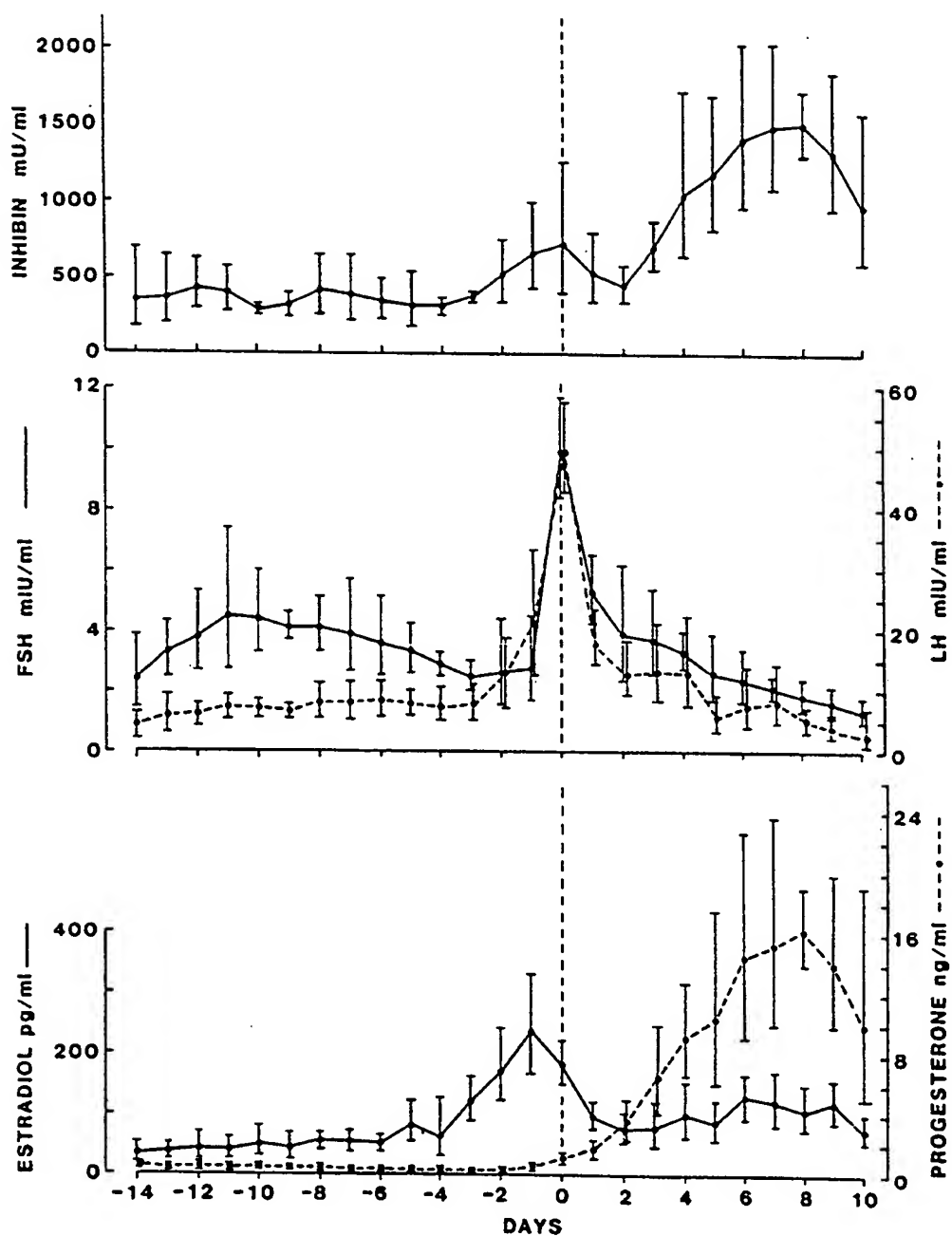
Fig. 14.



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Fig. 16.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 87/00070

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> ("Special classification symbols apply, indicate all") According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl. <sup>4</sup> G01N 33/541, 33/74, C07K 15/12				
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched:				
Classification System	Classification Symbols			
IPC	Derwent World Patent Index, Keyword "INHIBIN"			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched:				
AU: IPC G01N 33/53, 33/577, 33/541, 33/54, 33/74, C07G 7/00, C07K 15/12				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category * 1	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **		
X	International Journal of Gynaecology and Obstetrics, Volume 21, No.6, issued 1983 (Limerick, Ireland), S.P. Dandekar et al, "Levels of immunoreactive inhibin-like material in urine during the menstrual cycle", see pages 443-450.	ALL		
X	S.M. McCann et al (Editors), Role of Peptides and Proteins in Control of Reproduction, Proceedings Workshop 1982, published 1983 by Elsevier (New York), F.H. DeJong et al, "Assay and Purification of Inhibin" see pages 257-273, especially pages 269-272.	ALL		
X	Molecular and Cellular Endocrinology, Volume 44, No.3, issued 1986, March (Limerick, Ireland), D.M. Robertson et al, "Isolation of a 31 kDa form of inhibin from bovine follicular fluid", see pages 271-277, especially pages 273-276.	ALL		
X,P	Molecular and Cellular Endocrinology, Volume 46, No.2, issued 1986, July (Limerick, Ireland), R.I. McLachlan et al, "The radioimmunoassay of bovine and human follicular fluid and serum inhibin", see pages 175-185.	ALL		
X,P	WO,A, 86/06076 (BIOTECHNOLOGY AUSTRALIA PTY LTD et al) 23 October 1986 (23.10.86) see especially pages 47-53	ALL		
continued				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; border: none;">           * Special categories of cited documents: 10            "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier document but published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 50%; vertical-align: top; border: none;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.            "Z" document member of the same patent family         </td> </tr> </table>			* Special categories of cited documents: 10 "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family
* Special categories of cited documents: 10 "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family			
<b>IV. CERTIFICATION</b>				
Date of the Actual Completion of the International Search 22 June 1987 (22.06.87)		Date of Mailing of this International Search Report (07.07.87) 7 JULY 1987		
International Searching Authority Australian Patent Office		Signature of Authorized Officer J.G. HANSON		

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	WO/A, 86/00078 (MONASH UNIVERSITY et al) 3 January 1986 (03.01.86)	ALL
X	Biochemical and Biophysical Research Communications, Volume 133, No.1, issued 1985, November 27 (New York, New York), J. Rivier et al, "Purification and partial characterization of inhibin from porcine follicular fluid", see pages 120-127.	1,2,5
X,P	Biochemical and Biophysical Research Communications, Volume 136, No.3, issued 1985, May 14 (New York, New York), K. Miyamoto et al, "Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin", see pages 1103-1109.	1,3,4,7

continued

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Journal of Endocrinology, Volume 109, No.3, issued 1986, June (Colchester, Essex, England), V. Lee et al, "Monoclonal antibody to rat ovarian inhibin", see pages 379-383.	1,4,5,7
X,P	Journal of Endocrinology, Volume 111, No.2, issued 1986, November (Colchester, Essex, England), S. Van Dijk et al, "Sexual dimorphism in immunoneutralization of rat and ovine inhibin", see pages 255-261.	1,2,3,5
X,E	AU,A, 63512/86 (GENENTECH INC.) 9 April 1987 (09.04.87) see page 50.	1,2,7
X	Advances in Experimental Medicine and Biology, Volume 147, issued 1982 (New York, New York), F.H. De Jong et al, "Purification, characterization, and in vitro production of inhibin", see pages 37-52 especially pages 42-46.	1,2,3,5,6,7
X	Biological Research in Pregnancy and Perinatology, Volume 4, No.3, issued 1983 (Munich-Deisenhofen, West Germany), J.J. Sheth et al, "Bioimmunoreactive inhibin-like substance in human fetal gonads", see pages 110-112.	1,2,3,7,8,9
X	Journal of Biosciences, Volume 7, No.2, issued 1985, March (India), A.H. Bandivdekar et al, "Isolation of inhibin-like peptides from human placenta", see pages 175-190.	1,2,3,5
X	Chemical Abstracts, Volume 102, No.17, issued 1985, April 29 (Columbus, Ohio, USA) X. Lu et al, "Preparation of inhibin from porcine semen and its radioimmunoassay", see page 64, column 1, abstract No.143 300h, Shengzhi Yu Biyun, 1984 4(4), 27-33 (Chin)	1,2,7
X	Indian Journal of Experimental Biology, Volume 23, No.10, issued 1985, October (New Delhi, India), K.S. Hurkadli et al, "Studies on immunoneutralization of inhibin: a time course study", see pages 561-565.	1,2,3,5,7
X,P	US,A, 4624944 (LI et al) 25 November 1986 (25.11.86) see columns 7-9.	1,2,3,7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON  
INTERNATIONAL APPLICATION NO. PCT/AU 87/00070

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members			
AU 63512/86		EP 222491	IL 80218		
WO 8606076		AU 59039/86 IL 78519	DK 6113/86	EP 218717	
WO 8600078		AU 44374/85 IL 75412	DK 605/86 NO 860427	EP 185034 ZA 8504346	

END OF ANNEX